IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Ming-Bo Wang et al.	Group Art Unit: 1635
Application No.: 09/287,632	Examiner: JANE J ZARA
Filed: April 7, 1999	Confirmation No.: 6526
For: METHODS AND MEANS FOR OBTAINING MODIFIED PHENOTYPES	

DECLARATION BY INVENTOR UNDER 37 C.F.R. § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

We, Peter Michael Waterhouse, Ming-Bo Wang, Michael Wayne Graham and Neil A. Smith, citizens of Australia, hereby state as follows:

1. We are the named inventors of the subject matter of the above captioned application.

2. We have read and understood the Office Action dated November 1, 2007. We understand that U.S. Patent No. 6,506,559 has been cited in the Office Action in rejecting the claims of the present application.

3. We submit Exhibits 1-5 as evidence that embodiments of the presently claimed invention were made, i.e. actually reduced to practice, prior to the December 23, 1997 filing date of U.S. Provisional Application No. 60/068,562 of which benefit is claimed by U.S. Patent No. 6,506,559.

4. Exhibit 1 is a copy of 7 pages from a laboratory notebook prepared by Neil Smith that recorded experiments performed by Neil Smith and/or under his supervision during the construction of a chimeric DNA comprising two copies of a 0.75 kb fragment of the cDNA copy

of the Potato Virus Y RNA genome (PVY) in inverted orientation under control of a CaMV 35S promoter and further comprising intron 2 of the *Pdk* gene from *Flaveria trinervia* in the transcribed region of the chimeric DNA.

5. Notebook page 151 (Exhibit 1, page 1) describes the relevant part of expression vector N2, which was used as starting material to produce the chimeric DNA as described in paragraph 4. That N2 is an expression vector can be seen from the notation ART7, which indicates the backbone of the expression vector pART7 which was commonly used within the CSIRO Plant Industry laboratories at that time. The figure indicates the restriction enzyme sites and denotes the different functional parts of the N2 vector as blocks. The bent arrows indicate the promoter orientation, while the straight arrows indicate the orientation of the PVY inserts. The sizes of the functional parts are indicated between brackets below the blocks and are noted as kilobasepairs. Abbreviations used are: 35S: Cauliflower Mosaic Virus 35S promoter; PVY: approximately 0.75kb fragment of the sequence encoding the protease of potato virus Y; S4: 0.6 kb fragment of the Subterranean Clover Stunt Virus segment S4; Ocs3': 3' termination and polyadenylation signal from the octopine synthase gene from *Agrobacterium tumefaciens*.

6. The notebook page 151 further indicates a first cloning strategy to produce a PVY hairpin RNA having complementary sense and antisense strands targeting the PVY genome part, whereby the 0.6kb HindIII fragment comprising the Subterranean Clover Stunt Virus segment S4 fragment was deleted. In the product, both copies of the PVY fragment are operably linked under the control of one CaMV35S promoter. Upon transcription from the CaMV35S promoter which is active in plant cells, a double stranded RNA molecule as schematically represented ("mRNA double stranded") would be produced. The RNA molecule as represented is an artificial hairpin RNA structure comprising two annealing RNA sequences labeled "sense strand" and "α sense strand", wherein one of the annealing RNA sequences comprises a sense sequence of 750 nucleotides identical to 750 consecutive nucleotides of a PVY target gene of interest in a eukaryotic cell, in this case a plant cell, and the second of the annealing RNA sequences comprises an antisense sequence identical to 750 consecutive nucleotides of the complement of the PVY target gene of interest.

7. Notebook pages 186-188 (Exhibit 1, pages 2-4) describe the preparation of intron 2 of the pdk gene of *Flaveria trinervia* and cloning of a PCR amplified fragment having intron 2 (767bp) flanked by restriction enzymes into a small cloning vector (PBC) for amplification purposes.

8. On Notebook page 186 plasmid pdk1 is described comprising a 10.5 kb EcoRI fragment of the pdk genomic DNA in pBLUESCRIPT vector. The figure is a schematic representation of the pkdA genomic DNA, the cloned EcoRI fragment and the position of intron 2. At the bottom is a photo of diagnostic restriction enzyme digests to confirm the structure of pdk1.

9. Notebook page 187 represents a nucleotide sequence alignment of the pdkA and pdkB genes with an indication of the position and nucleotide sequence of intron 2 of pdkA. Further indicated are the nucleotide sequences and position of the oligonucleotides used as PCR primers to specifically amplify the intron 2 fragment, flanked by HindIII restriction enzyme recognition sites.

10. Notebook page 188 details the PCR reaction protocol to amplify the intron 2 fragment from the plasmid pdk1, a gel analysis of the amplified fragment, a ligation protocol for introduction of the PCR- amplified, HindIII restricted product into a small vector named PBC and determination of the relative orientation of insertion of the HindIII fragment comprising the intron 2 by restriction analysis. At the bottom of the page, the two alternative orientations of insertion into PBC are schematically represented, together with an indication of the expected size of the fragment which was generated after BglII/PstI restriction digestion for both orientations and indication of the orientation of inserts in the different clones.

11. The first picture of the gel on Notebook page 189 concerns additional verification for the clones of the intron 2 HindIII fragment in PBC in both orientations. Immediately below the

picture is a schematic representation of the cloning strategy to introduce the intron 2 between the two fragments of PVY in inverted orientation under control of a CaMV35S promoter, to generate a double stranded RNA-encoding chimeric gene. To this end, the 0.6 kb HindIII S4 fragment from T-DNA vector pNS2 was replaced by the 0.77 kb HindIII fragment comprising the intron 2 described under paragraph 10. pNS2 comprised the gene components of expression vector N2 described on notebook page 151 (discussed in paragraph 5 above) cloned into T-DNA vector pART27 (see Exhibit 1, page 6 where it is indicated that the backbone vector is pART27, a T-DNA vector commonly used at CSIRO for introduction of chimeric DNAs into plant cells by Agrobacterium-mediated transformation). Notebook page 189 further describes the composition of the ligation mix and the restriction enzyme analysis to identify clones in which the S4 fragment had been replaced by the intron 2 fragment. A small table at the bottom of notebook page 189 indicates the predicted sizes of fragments generated by restriction enzyme digests for each of the two possible orientations of the inserted intron fragment (indicated as "sense intron" and "α-sense intron", the latter is shorthand for intron in antisense orientation).

12. Notebook page 192 (Exhibit 1, page 6) displays the restriction enzyme analysis with ApaI enzyme to verify which of the candidate clones contained a single insert of the intron fragment. As indicated next to the gel, the clones with a single insert were expected to produce a restriction fragment of 770 bp, and as stated there "appear to be many [candidate clones] with single Intron 2 inserts". The potentially good chimeric DNA constructs were further verified through XbaI restrictions enzyme analysis which as indicated were expected produce a characteristic 2.3 kb fragment. At least candidates "2 and 6 (from pool 7) appear to have [the] intron insert".

13. The bottom part of the Notebook page 192 then represents the restriction enzyme analysis with the restriction enzyme combinations indicated at the bottom of page 189 to determine the orientation of the intron 2 in the chimeric DNA constructs for 4 different clones. The notification under the gel pictures indicate that clone "2" had the intron in antisense orientation, while clones "6", "original single clone 1" and clone "6 from pool 7" had the intron fragment in the sense orientation. Intron 2 is a heterologous intron with respect to the PVY inserts in the chimeric DNA.

14. Notebook page 193 (Exhibit 1, page 7) represents a quality control of a large scale DNA preparation of the T-DNA vector clones 2 and 6 from Notebook page 192. At the top of page 193 it is indicated that the chimeric DNA construct with the intron fragment in antisense orientation is named pNS9, while the chimeric DNA construct with the intron fragment in sense orientation is named pNS10. The bullet point "set up 2 and 6 for tri-parental => LBA4404" refers to the introduction of T-DNA vectors from the E. coli host into Agrobacterium tumefaciens comprising a disarmed helper Ti-plasmid by a process referred to as triparental mating commonly used in the art. The resulting agrobacterial transconjugant strains were cocultivated with tobacco W38 cells in order to introduce the chimeric DNA into plant cells.

15. Exhibit 1 thus describes the successful construction of a chimeric DNA construct molecule comprising, in order:

a) a promoter, operative in a eukaryotic (plant) cell (*CaMV35S*);

b) a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising

i. a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the nucleotide sequence of the nucleic acid of interest (*0.75 kb PVY region in sense orientation- the nucleic acid of interest is thus comprised in the genome of an infecting RNA virus in this embodiment)*); and

ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (*0.75 kb PVY region in antisense orientation*);

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequences; and

wherein the DNA region further comprises an intron (*intron 2*) (which is a heterologous intron with respect to the sense and antisense PVY sequences); and

c) a DNA region involved in transcription termination and polyadenylation (*3' ocs region*).

16. The experiments recorded in Exhibit 1 were completed prior to December 23, 1997.

17. The experiments recorded in Exhibit 1 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

18. Exhibit 2 is a copy of 5 pages from a laboratory notebook (Notebook No 5) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision during the construction of a chimeric DNA encoding an RNA comprising sense and antisense nucleotide sequences targeted to a β-glucuronidase gene (GUS) in which the sense and antisense nucleotide sequences could basepair over about 558 bases, as described in the above mentioned US patent application in Example 1, Figure 1A (6) and Sequence Listing entry SEQ ID NO 1. Further recorded in Exhibit 2 are the introduction of the chimeric gene into a T-DNA vector and introduction of the resulting T-DNA vector into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid. The introduction of the chimeric DNA into plant cells, and data showing reduction of target gene expression in the plant cells are recorded in Exhibits 4 and 5.

19. Exhibit 2, page 1, entitled "To make invert-repeat GUS construct for rice transformation" recorded the starting materials and ligation mixes set up to introduce a second copy of part of the GUS coding region into a vector pWUJGdT comprising a GUS coding region with a deletion of the internal 0.3kb EcoRV fragment.

20. Two cloning strategies were followed i.e.

a. to introduce a HincII fragment from the GUS coding region derived from vector pWJKKGUS (indicated on top of the page as DNA 3) into EcoRI restricted vector pWUJGdT (indicated on top of the page as DNA 1 –

treated to render the sticky ends of the restriction fragment blunt and further treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page. This ligation mix is recorded as ligation 2.

b. To introduce a SmaI-EcoRV fragment from the GUS coding region derived from vector pWJKKGUS (indicated on top of the page as DNA 4) into SmaI restricted vector pWUJGdT (indicated on top of the page as DNA 2 – treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page). This ligation mix is recorded as ligation 4.

21. Exhibit 2, page 2 recorded the identification of candidate clones resulting from the cloning strategies, as well as identification of the orientation of the insert into the vector. At the bottom of the page, these two possible insert orientations into the chimeric DNA are schematically represented, resulting in two different vectors namely:

a. candidate clone 2 (2) renamed pMBW234 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional β-glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the same orientation as the GUSd coding region, followed by transcription termination and polyadenylation region from Agrobacterium T-DNA gene *tml* (tml').

b. candidate clone 2 (4) renamed pMBW233 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional β-glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the inverse orientation relative to the GUSd coding region, followed by transcription termination and polyadenylation region from Agrobacterium T-DNA gene *tml* (tml'). Transcription of this chimeric gene yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences, namely a sense RNA sequence identical to about 558 consecutive nucleotides of the GUS

gene and an antisense RNA sequence identical to about 558 consecutive nucleotides of the complement of the GUS gene.

22. Exhibit 2, page 3 records the experiments performed to clone the chimeric genes described under paragraph 21, as NotI restriction fragments, into a T-DNA vector designated binary vector pWBVec4A. As indicated in the top right insert, "ligation 2" uses "DNA 1", i.e NotI fragment from pMBW234 ; "ligation 3" uses "DNA 2" i.e NotI fragment from pMBW233.

23. Exhibit 2, page 4 contains the continued recorded data from the experiments described under paragraph 22, including the restriction enzyme analysis, of candidate clones from ligations 2 and 3 mentioned on the previous page. The plasmid denoted pMBW237 refers to the chimeric gene in colony 2(8) [clone 8 of ligation mix 2, thus corresponding to a chimeric gene as in pMBW234], and the plasmid denoted pMBW239 refers to the chimeric gene in colony 3(8) [clone 8 of ligation mix 3, thus corresponding to a chimeric gene as in pMBW233].

24. At the bottom of Exhibit 2, page 4 it is indicated that T-DNA vectors 2(8) and 3(8) were used for triparental mating, referring to the introduction of the T-DNA vectors from the E. coli host into Agrobacterium tumefaciens comprising a disarmed helper Ti-plasmid, and Exhibit 2, page 5 records the verification of the Agrobacterium transconjugants as containing the respective T-DNA vectors.

25. The experiments recorded in Exhibit 2 were completed prior to December 23, 1997.

26. The experiments recorded in Exhibit 2 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

27. Exhibit 3 is a copy of a publication by Alan H. Christensen and Peter H. Quail, entitled "Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants", which was published in *Transgenic Research* Volume 5, 213-218 (May 1996). This publication describes the ubiquitin promoter region fragment used in the construction of the chimeric genes described in Exhibit 2, as explained under paragraph 21. As indicated in Figure 1, the Ubi promoter region included an intron (Ubi-1 intron) indicated in the Figure by an angled line) in the transcribed region. This publication is mentioned in the above mentioned patent application as a reference for the Ubiquitin promoter used in the chimeric constructs described in Example 1 of that patent

application. At the time of the construction of the chimeric genes, described under paragraphs 20 and 21, we were aware of the presence of an intron in the transcribed DNA region under the control of the Ubi promoter region.

28. Exhibit 4 is a copy of 6 pages from a laboratory notebook (Notebook 96) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision to transform transgenic rice calli containing and expressing a recombinant GUS gene as a target gene of interest in a eukaryotic (plant) cell, with the T-DNA vectors described in Exhibit 2, and to analyse phenotypic expression of GUS in the supertransformed rice calli comprising the target gene and the chimeric DNAs. Data from these analyses correspond to the data recorded in the patent application as "Example 1".

29. Exhibit 4, page 1 indicates that calli were initiated from transgenic rice seeds 4R-V10-28 and 4R-V10-67, both expressing a recombinant GUS gene of interest integrated in the genome of the calli cells. This target gene is a transgene in the cells. The bottom half indicates that these calli were "supertransformed" using different Agrobacteria comprising the different T-DNA vectors including pMBW239 (experiment indicated as "7"). Note also the control experiments where an "empty" control T-DNA vector (pMBW223) was used (experiment 1) and where a conventional co-suppression construct (comprising a transcribed sense nucleotide sequence of the GUSd gene (pMBW225) was used for comparison of the level of phenotypic expression of the target gene (experiment 2).

30. Exhibit 4, page 2 recorded the data for Bialaphos resistant calli obtained with the different Agrobacteria described on the previous page (paragraph 24 above), indicating that such calli had been transformed by the T-DNA's including the chimeric DNA silencing constructs (or control constructs). The transgenic calli obtained by transformation of the initial V10-28 transgenic rice cells with pMBW239 are indicated as "7a" whereas the transgenic calli obtained by transformation of the initial V10-67 transgenic rice cells with pMBW239 are indicated as "7b".

31. Exhibit 4, page 2 at the bottom and continuing on page 3, recorded the data of an initial GUS staining experiment on parts of the transgenic calli that were obtained (V10-67 derived). In control experiment "1b" where the GUS transgenic calli were supertransformed by an "empty" T-DNA vector, all supertransformed calli strongly stained blue indicating strong GUS activity. On page 3 of Exhibit 4, the results of a similar GUS staining on calli obtained by supertransformation with pMBW239 indicating that the observed GUS staining was either weak

("W") , negative ("-") or resulted in an isolated blue spot. These data indicated that the chimeric DNA from pMBW239 reduced the phenotypic expression of the GUS target gene in the rice cells.

32. Exhibit 4, pages 4 and 5 represent the recordation of these data for the individual calli. Exhibit 4, page 6 summarizes the data of an initial GUS staining experiment on parts of the obtained transgenic calli (V10-28 derived). While in series 1a, all calli transformed by the control T-DNA vector pMBW223 strongly stained blue indicating strong GUS activity, the transgenic calli obtained by transformation with pMBW239 (series 7a) all scored as "very weak" or "basically GUS negative" with the exception of only two calli. On the bottom of page 6, reference is made to (quantitative) GUS assays, the data of which are recorded in Notebook 5, starting on page 55, discussed hereinafter as Exhibit 5.

33. The experiments recorded in Exhibit 4 were completed prior to December 23, 1997.

34. The experiments recorded in Exhibit 4 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

35. Exhibit 5 is a copy of 17 pages from a laboratory notebook (Notebook 5) prepared by Ming Bo Wang that recorded quantitative GUS analysis experiments performed by Ming-Bo Wang and/or under his supervision, on the supertranformed rice calli described in Exhibit 4. Particular attention is drawn to the "raw data" concerning GUS analysis by kinetic value measurements on Exhibit 5, page 14 which are represented in Example 1, Table 2 of the above mentioned patent application. See e.g. data for Plate No 3, wells 1A to 2H which have in handwriting indications "7a1" to "7a14" referring to transgenic lines, which are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-28. Similarly, data for Plate No 3, wells 3A to 4D which have in handwriting indications "7b2" to "7b16" referring to transgenic lines are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-67.

36. The experiments recorded in Exhibit 5 were completed prior to December 23, 1997.

37. The experiments recorded in Exhibit 5 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

38. The experiments recorded in Exhibit 2, 4 and 5 therefore describe successful completion prior to December 23, 1997 of the construction of a chimeric DNA molecule **(*pMBW233/239 series*)** comprising in order

a. a promoter operative in a eukaryotic (plant) cell (***Ubi-P***);

b. a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising

i. a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100 % sequence identity with at least 20 (and also at least 50 or 100 nucleotides) consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest (***Gusd in sense orientation***) in a eukaryotic cell; and

ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (***Gus5' in antisense orientation***);

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence wherein the DNA region further comprises an intron (***Ubi intron***) (which is heterologous to the sense GUS sequence); and

c. a DNA region involved in transcription termination and polyadenylation (***tml'***).

39. The experiments recorded in Exhibit 2, 4 and 5 also describe successful completion prior to December 23, 1997 of a method for reducing the phenotypic expression of a nucleic acid of interest which is normally capable of being expressed (**GUS gene**) in a eukaryotic cell (a plant cell, ***rice***) comprising the step of introducing into the eukaryotic cell (plant cell) a chimeric DNA **(*pMBW233/239 series*)** as described in paragraph 24.

40. The statements made in this declaration are made on the personal knowledge or on the information and belief of the declarants. We hereby declare that all statements made herein of personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 1st May 2008 Peter Waterhouse.
Peter Michael Waterhouse,

Date: 1st May 2008 Ming-Bo Wang
Ming-Bo Wang

Date: _____
Michael Wayne Graham

Date: 1st MAY 2008 Neil Smith
Neil A. Smith

Attorney's Docket No. 1021565-000060

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picture is a schematic representation of the cloning strategy to introduce the intron 2 between the two fragments of PVY in inverted orientation under control of a CaMV35S promoter, to generate a double stranded RNA-encoding chimeric gene. To this end, the 0.6 kb HindIII S4 fragment from T-DNA vector pNS2 was replaced by the 0.77 kb HindIII fragment comprising the intron 2 described under paragraph 10. pNS2 comprised the gene components of expression vector N2 described on notebook page 151 (discussed in paragraph 5 above) cloned into T-DNA vector pART27 (see Exhibit 1, page 6 where it is indicated that the backbone vector is pART27, a T-DNA vector commonly used at CSIRO for introduction of chimeric DNAs into plant cells by Agrobacterium-mediated transformation). Notebook page 189 further describes the composition of the ligation mix and the restriction enzyme analysis to identify clones in which the S4 fragment had been replaced by the intron 2 fragment. A small table at the bottom of notebook page 189 indicates the predicted sizes of fragments generated by restriction enzyme digests for each of the two possible orientations of the inserted intron fragment (indicated as "sense intron" and "α-sense intron", the latter is shorthand for intron in antisense orientation).

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ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (*0.75 kb PVY region in antisense orientation*);

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequences; and

wherein the DNA region further comprises an intron (*intron 2*) (which is a heterologous intron with respect to the sense and antisense PVY sequences); and

c) a DNA region involved in transcription termination and polyadenylation (*3' ocs region*).

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17. The experiments recorded in Exhibit 1 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

18. Exhibit 2 is a copy of 5 pages from a laboratory notebook (Notebook No 5) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision during the construction of a chimeric DNA encoding an RNA comprising sense and antisense nucleotide sequences targeted to a β-glucuronidase gene (GUS) in which the sense and antisense nucleotide sequences could basepair over about 558 bases, as described in the above mentioned US patent application in Example 1, Figure 1A (6) and Sequence Listing entry SEQ ID NO 1. Further recorded in Exhibit 2 are the introduction of the chimeric gene into a T-DNA vector and introduction of the resulting T-DNA vector into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid. The introduction of the chimeric DNA into plant cells, and data showing reduction of target gene expression in the plant cells are recorded in Exhibits 4 and 5.

19. Exhibit 2, page 1, entitled "To make invert-repeat GUS construct for rice transformation" recorded the starting materials and ligation mixes set up to introduce a second copy of part of the GUS coding region into a vector pWUJGdT comprising a GUS coding region with a deletion of the internal 0.3kb EcoRV fragment.

20. Two cloning strategies were followed i.e.

a. to introduce a HincII fragment from the GUS coding region derived from vector pWJKKGUS (indicated on top of the page as DNA 3) into EcoRI restricted vector pWUJGdT (indicated on top of the page as DNA 1 –

treated to render the sticky ends of the restriction fragment blunt and further treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page. This ligation mix is recorded as ligation 2.

b. To introduce a SmaI-EcoRV fragment from the GUS coding region derived from vector pWJKKGUS (indicated on top of the page as DNA 4) into SmaI restricted vector pWUJGdT (indicated on top of the page as DNA 2 – treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page). This ligation mix is recorded as ligation 4.

21. Exhibit 2, page 2 recorded the identification of candidate clones resulting from the cloning strategies, as well as identification of the orientation of the insert into the vector. At the bottom of the page, these two possible insert orientations into the chimeric DNA are schematically represented, resulting in two different vectors namely:

a. candidate clone 2 (2) renamed pMBW234 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional β-glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the same orientation as the GUSd coding region, followed by transcription termination and polyadenylation region from Agrobacterium T-DNA gene *tml* (tml').

b. candidate clone 2 (4) renamed pMBW233 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional β-glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the inverse orientation relative to the GUSd coding region, followed by transcription termination and polyadenylation region from Agrobacterium T-DNA gene *tml* (tml'). Transcription of this chimeric gene yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences, namely a sense RNA sequence identical to about 558 consecutive nucleotides of the GUS

gene and an antisense RNA sequence identical to about 558 consecutive nucleotides of the complement of the GUS gene.

22. Exhibit 2, page 3 records the experiments performed to clone the chimeric genes described under paragraph 21, as NotI restriction fragments, into a T-DNA vector designated binary vector pWBVec4A. As indicated in the top right insert, "ligation 2" uses "DNA 1", i.e NotI fragment from pMBW234 ; "ligation 3" uses "DNA 2" i.e NotI fragment from pMBW233.

23. Exhibit 2, page 4 contains the continued recorded data from the experiments described under paragraph 22, including the restriction enzyme analysis, of candidate clones from ligations 2 and 3 mentioned on the previous page. The plasmid denoted pMBW237 refers to the chimeric gene in colony 2(8) [clone 8 of ligation mix 2, thus corresponding to a chimeric gene as in pMBW234], and the plasmid denoted pMBW239 refers to the chimeric gene in colony 3(8) [clone 8 of ligation mix 3, thus corresponding to a chimeric gene as in pMBW233].

24. At the bottom of Exhibit 2, page 4 it is indicated that T-DNA vectors 2(8) and 3(8) were used for triparental mating, referring to the introduction of the T-DNA vectors from the E. coli host into Agrobacterium tumefaciens comprising a disarmed helper Ti-plasmid, and Exhibit 2, page 5 records the verification of the Agrobacterium transconjugants as containing the respective T-DNA vectors.

25. The experiments recorded in Exhibit 2 were completed prior to December 23, 1997.

26. The experiments recorded in Exhibit 2 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

27. Exhibit 3 is a copy of a publication by Alan H. Christensen and Peter H. Quail, entitled "Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants", which was published in *Transgenic Research* Volume 5, 213-218 (May 1996). This publication describes the ubiquitin promoter region fragment used in the construction of the chimeric genes described in Exhibit 2, as explained under paragraph 21. As indicated in Figure 1, the Ubi promoter region included an intron (Ubi-1 intron) indicated in the Figure by an angled line) in the transcribed region. This publication is mentioned in the above mentioned patent application as a reference for the Ubiquitin promoter used in the chimeric constructs described in Example 1 of that patent

application. At the time of the construction of the chimeric genes, described under paragraphs 20 and 21, we were aware of the presence of an intron in the transcribed DNA region under the control of the Ubi promoter region.

28. Exhibit 4 is a copy of 6 pages from a laboratory notebook (Notebook 96) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision to transform transgenic rice calli containing and expressing a recombinant GUS gene as a target gene of interest in a eukaryotic (plant) cell, with the T-DNA vectors described in Exhibit 2, and to analyse phenotypic expression of GUS in the supertransformed rice calli comprising the target gene and the chimeric DNAs. Data from these analyses correspond to the data recorded in the patent application as "Example 1".

29. Exhibit 4, page 1 indicates that calli were initiated from transgenic rice seeds 4R-V10-28 and 4R-V10-67, both expressing a recombinant GUS gene of interest integrated in the genome of the calli cells. This target gene is a transgene in the cells. The bottom half indicates that these calli were "supertransformed" using different Agrobacteria comprising the different T-DNA vectors including pMBW239 (experiment indicated as "7"). Note also the control experiments where an "empty" control T-DNA vector (pMBW223) was used (experiment 1) and where a conventional co-suppression construct (comprising a transcribed sense nucleotide sequence of the GUSd gene (pMBW225) was used for comparison of the level of phenotypic expression of the target gene (experiment 2).

30. Exhibit 4, page 2 recorded the data for Bialaphos resistant calli obtained with the different Agrobacteria described on the previous page (paragraph 24 above), indicating that such calli had been transformed by the T-DNA's including the chimeric DNA silencing constructs (or control constructs). The transgenic calli obtained by transformation of the initial V10-28 transgenic rice cells with pMBW239 are indicated as "7a" whereas the transgenic calli obtained by transformation of the initial V10-67 transgenic rice cells with pMBW239 are indicated as "7b".

31. Exhibit 4, page 2 at the bottom and continuing on page 3, recorded the data of an initial GUS staining experiment on parts of the transgenic calli that were obtained (V10-67 derived). In control experiment "1b" where the GUS transgenic calli were supertransformed by an "empty" T-DNA vector, all supertransformed calli strongly stained blue indicating strong GUS activity. On page 3 of Exhibit 4, the results of a similar GUS staining on calli obtained by supertransformation with pMBW239 indicating that the observed GUS staining was either weak

("W") , negative ("-") or resulted in an isolated blue spot. These data indicated that the chimeric DNA from pMBW239 reduced the phenotypic expression of the GUS target gene in the rice cells.

32. Exhibit 4, pages 4 and 5 represent the recordation of these data for the individual calli. Exhibit 4, page 6 summarizes the data of an initial GUS staining experiment on parts of the obtained transgenic calli (V10-28 derived). While in series 1a, all calli transformed by the control T-DNA vector pMBW223 strongly stained blue indicating strong GUS activity, the transgenic calli obtained by transformation with pMBW239 (series 7a) all scored as "very weak" or "basically GUS negative" with the exception of only two calli. On the bottom of page 6, reference is made to (quantitative) GUS assays, the data of which are recorded in Notebook 5, starting on page 55, discussed hereinafter as Exhibit 5.

33. The experiments recorded in Exhibit 4 were completed prior to December 23, 1997.

34. The experiments recorded in Exhibit 4 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

35. Exhibit 5 is a copy of 17 pages from a laboratory notebook (Notebook 5) prepared by Ming Bo Wang that recorded quantitative GUS analysis experiments performed by Ming-Bo Wang and/or under his supervision, on the supertranformed rice calli described in Exhibit 4. Particular attention is drawn to the "raw data" concerning GUS analysis by kinetic value measurements on Exhibit 5, page 14 which are represented in Example 1, Table 2 of the above mentioned patent application. See e.g. data for Plate No 3, wells 1A to 2H which have in handwriting indications "7a1" to "7a14" referring to transgenic lines, which are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-28. Similarly, data for Plate No 3, wells 3A to 4D which have in handwriting indications "7b2" to "7b16" referring to transgenic lines are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-67.

36. The experiments recorded in Exhibit 5 were completed prior to December 23, 1997.

37. The experiments recorded in Exhibit 5 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

38. The experiments recorded in Exhibit 2, 4 and 5 therefore describe successful completion prior to December 23, 1997 of the construction of a chimeric DNA molecule (***pMBW233/239 series***) comprising in order

a. a promoter operative in a eukaryotic (plant) cell (***Ubi-P***);

b. a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising

i. a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100 % sequence identity with at least 20 (and also at least 50 or 100 nucleotides) consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest (***Gusd in sense orientation***) in a eukaryotic cell; and

ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (***Gus5' in antisense orientation***);

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence wherein the DNA region further comprises an intron (***Ubi intron***) (which is heterologous to the sense GUS sequence); and

c. a DNA region involved in transcription termination and polyadenylation (***tml'***).

39. The experiments recorded in Exhibit 2, 4 and 5 also describe successful completion prior to December 23, 1997 of a method for reducing the phenotypic expression of a nucleic acid of interest which is normally capable of being expressed (**GUS gene**) in a eukaryotic cell (a plant cell, ***rice***) comprising the step of introducing into the eukaryotic cell (plant cell) a chimeric DNA (***pMBW233/239 series***) as described in paragraph 24.

40. The statements made in this declaration are made on the personal knowledge or on the information and belief of the declarants. We hereby declare that all statements made herein of personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: _____ _____
Peter Michael Waterhouse,

Date: _____ _____
Ming-Bo Wang

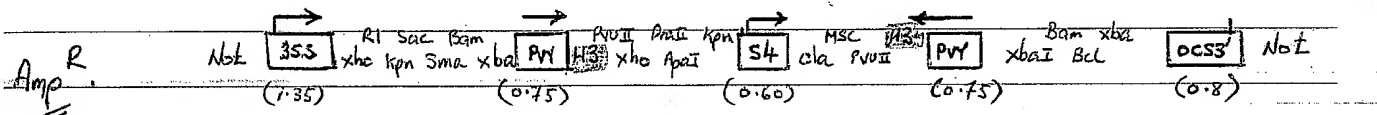
Date: 2 May, 2006 _____
Michael Wayne Graham

Date: _____ _____
Neil A. Smith

Experiment:..... WEDNESDAY.... Date_

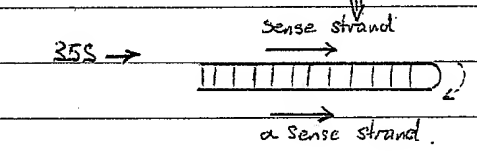
CREATION OF PVY mRNA HAIRPIN ("DOUBLE STRANDED")

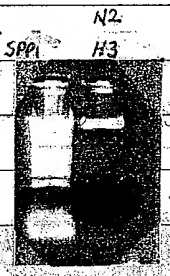
USING: N2 p 82 & 96



* Cut 10µl of N2 with H3 O/N 37°C

Will religate in 100µl to delete S4 promoter





LIGATION

VECTOR:	20µl digest	R.T.
BUFFER:	2.0µl	
H_2O:	62.0µl	
LIGASE:	3.0µl	
ATP(5mM):	5.0µl	
TOTAL:	100.µl	

No Colonies Rpt'd

Rpt Cut 10µl N2 with H3 for 2 hours. (200µl Reaction)
Resuspended in 10µl dH_2O. Ran 1ul

* No clones - All still have insert
Will repeat by cutting band from TAE Agarose & ligating.

LIGATION: Rpt

Cut Plasmid:	9.0µl
Buffer:	2.0µl
dH_2O:	7.0µl
Ligase:	1.0µl
ATP (5mM):	1.0µl
TOTAL	20.0µl

Zapped → DH5α cells
1µl → JM109 cells

Signature of Researcher ..... CONT' PAGE 154 ..... Date .....

Signature of Supervisor Neil Smith ..... Date .....

Experiment:..... WEDNESDAY Date _ _ _ _

PREPARATION OF INTRON (2) FROM FLAVARIA trauernia

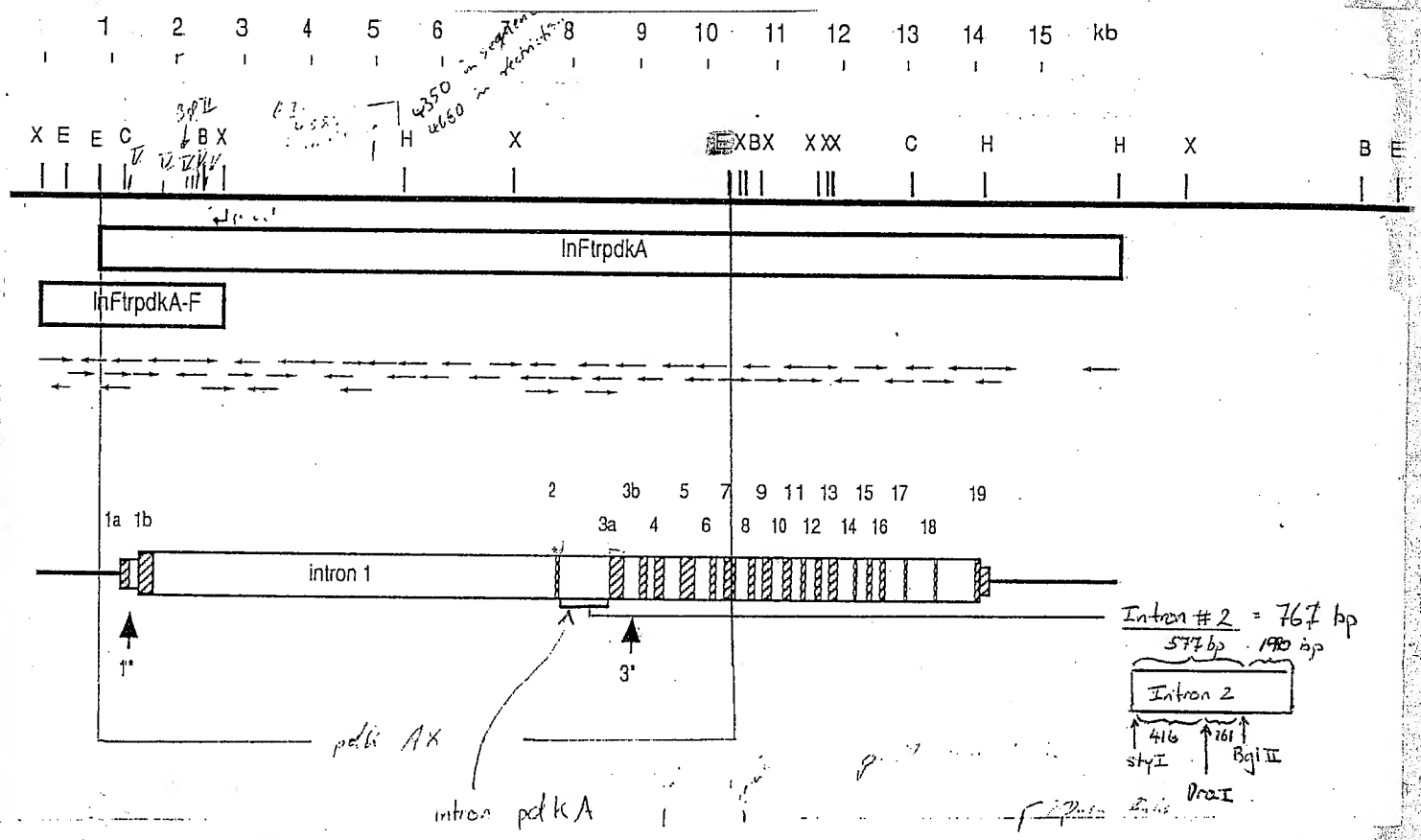
Plasmid provided by Bell Taylor

PdkA = Flaveria trauernia

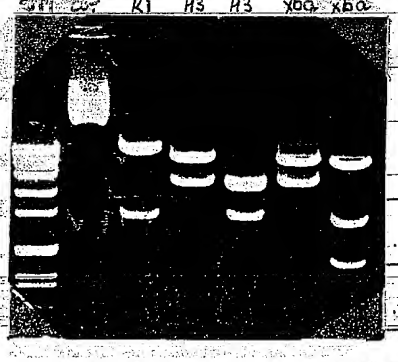
Pdk B = Flaveria brownii

pdk1 - 13.75 kb plasmid

Intron is in pBluescript (ks) as an EcoRI fragment (10.5 kb) - T3-T7 orientation



Set up Qiagen prep + dbd diagnostic cuts

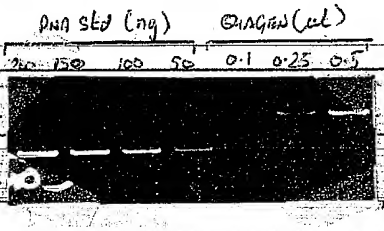


✓ R1 - 10.5kb (insert) + 3.0kb (Vector)

✓ H3 5.5 kb (5' end intron) + 7.5 (Rest)

✓ R1/H3 " " 4.5kb (3'end) + 3.0kb (Vector)

✓ xba ~4-4.5kb (centre)



R1/xba -

= 200ng/ul

Nad Smith

Witness

potentieller Translationsstart

Exon 2

```
pdkA  - ATTGAATAAAAAA---CATTTGATTTTGACTTTGTGTTTGATTGATTATG -6624
pdkB  - ATTGAAAGAAAAAAACCATTTGATTTTGACTTTGTGTTTGATTGCATATG -2868
pdkA  - CAGAGGGTTTTCACTTTTGGTAAAGGAAGAAGTGAAGGCAACAGGGACAT -6674
pdkB  - CAGAGGGTTTTCACTTTTGGTAAAGGAAACAGTGAAGGCAACAAGGACAT -2918
pdkA  - GAAATCCTTGGTAAGGAAATAATTATTTTCTTT--TTTCCTTTTAGTATA -6722
pdkB  - GAAATCCTTGGTAAGGAAATCATTATTTCCTTTCCTTTAATTATAATAGT -2968
pdkA  - AAATA----GTTAAGTGATGTTAATTAGTATGATTATAATAATATAGTTG -6768
pdkB  - AAGTAATGTGTTAAGTAATGTTAATTAGCATGATTATTATAATATGATTG -3018
pdkA  - TTATAATTGTGAAAAAATAATTTATAAATATATTGTTTACATAAACAACA -6818
pdkB  - TTATAATTGTGAAAAAG-AATTTATAAATATATTGTTTAGATAAACAACT -3067
pdkA  - TAGTAATGTAAAAAAATATGACAAGTGATGTGTAAGACGAAGAAGATAAA -6868
pdkB  - TACTAAGGTAAAT--TTATAACAAGTGATGTGTAATCCTAAGAAGATAAA -3115
pdkA  - AG-TTGAGAGTAAGTATATTATTTTTTAATGAATTTGATCGAACATGTAAG -6917
pdkB  - AAATTAAAAGTAAATATATT---TTTAATAAATTTGA----ACATATAAG -3158
pdkA  - ATGA-TA-TACTAGCATTAATATTTGTTTTAATCATAATAGTAAT----- -6960
pdkB  - ATGACTAATAATAACATTAATATTTGTTTTAATCATAATAGTAATAGTAA -3208
pdkA  - -TCTAGCTGGTTTGATGAATTAAATATCAATGATAAAATACTATAGTAAA -7009
pdkB  - TTCTAGTTGGTTTGATGAATTAAATA-CAATGATAAAGTACTATAGTAAA -3257
pdkA  - AATAAGAATAAATAAATTAAAATAATATTTTTTTATGATTAATAGTTTAT -7059
pdkB  - AATAAGAATAAGTAAATTAAAATAATATT-----ATGATTAGTAGTTTAT -3302
pdkA  - TATATAATTAAATATCTATACCATTACTAAATATTTTAGTTTAAAAGTTA -7109
pdkB  - TAGATAATTAAATATCTATACCATTACTAAATATTTTAGTATAAAAGTTA -3352
pdkA  - ATAAATATTTTGTTAGAAATTCCAATCTGCTTGTA--ATTTATCAATAAA -7157
pdkB  - ATAAATATTTTGTTTGAAATTCCAATCTGCTTGTATAATTTATCAATAAA -3402
pdkA  - CAAAATATTAAATAACAAGCTAAAGTAACAAATAATATCAAACTAATAGA -7207
pdkB  - CAAAATTTTAAATAACAAGCTAAAGTAACAAATAATATCAAACTAATAAA -3452
pdkA  - AACAGTAATCTAATG------TAACAAAAC---------- -7231
pdkB  - AACAGTAATCTAATGCTAATATAACAAAACGCAACGTCTATCAATTTTAT -3502
pdkA  - ATAATCT----------AATGCT--- -7244
pdkB  - ATAATATTATTTTTAATTAACATTCTTATTAATTTCTAAATAATACTTGT -3552
pdkA  - ----------
pdkB  - AGTATTACCATTTATCACCATAATAATTATTGATGAACTACTTATGTCAA -3602
pdkA  - AATATAAC----------AAAGCGCAAGA----------TCTATCA -7270
pdkB  - AATATAAATAAAAATAAATAAATTGCAATAGAATTTTAAACATTCCATCC -3652
pdkA  - TTTT----------ATAT---------- -7278
pdkB  - TTTTTTTACTAATGTATTTGGAATTCACTAATGGATATGAATCCTTATTA -3702
pdkA  - ----------
pdkB  - TTAATGAATTTAAAGCAACTCATAGTGAGCGTTTGGTTCGAAGGAATGGA -3752
pdkA  - ----------AGTATTATTTTCAA---------- -7292
pdkB  - ATGGAATGGAACGGAATTGAGAAACTAGTATAATTTTCAAGTAAAATTAT -3802
pdkA  - ----------TCAAC----A -7298
pdkB  - ATTTGAGTGGTTAATGAAATGTAAAACCCAAGGGAATGTTTCACCTCATA -3852
pdkA  - TTC----------TTATTAATTT---------- -7311
pdkB  - TTCCCTTGGTTGTGGGATTTTTGATTTTCCACCTAGGGAGGGAATTTGAA -3902
```

```
pdkA  - ----------
pdkB  - ATCACACAACCAAGAGAATTTGAGGTGAACATTCCCTTGGGTTTTACATT -3952
pdkA  - ----------CTAAA--TAAT---ACTTG----------TAGTTT---- -7331
pdkB  - TCATTACCCACCTAAACATAATTTTATTTGAAAATTATATTAGTTTCTCA -4002
pdkA  - --------------TATT------AAC----------TTCT -7342
pdkB  - ATTCCATTACATTTTATTCCTTCCAACCAAACGCTCCCTATAGTGTTTGT -4052
pdkA  - ----------AAATGGATTGACTATTAATTAAATGAATTAGTCGAACAT -7381
pdkB  - TTGGTTGGGTGAAATTGATTGACAAATAATTAAATGAACAAG--GTAATA -4100
pdkA  - GAATAAACAAGG------TAACATGATAGATCATGTCATTGTGTTATCAT -7425
pdkB  - GAATCAACAAGACATGAATAACATGATAGATCACTTCATTCTGTTATCAT -4150
pdkA  - TGATCTTACATTTGGATTGATTACAGTTGGGAGGAAAAGGAGCAAATCTT -7475
pdkB  - TGATCTTACATTTGGATTGATTACAGTTGGGAGGAAAAGGTGCAAATCTT -4200
pdkA  - GCTGAGATGTCAAGCATTGGTCTATCAGTTCCTCCTGGGCTCACTATTTC -7525
pdkB  - GCAGAGATGGCAAGCATTGGCCTATCAGTTCCTCCTGGGCTCACTATTTC -4250
```

Designed primers

Intron 2 (5')

Intron 2

5' CGGTACATGAAAAGCTTGGTAAGC 3'

Hind III

23 mer - 11 GC

12 TA oligo 193 ng/µl

Intron 2 (3') LABELLED "Intron 2 (B)"

Hind III

3' AG 5'

GCTAACTAATGTCAAACCCTTCGAAAGGA

A

Intron 2

Complementary strand 28 mer

oligo = 245 ng/µl

P.T.O

Na Smith Date ___/___/___

Date ___/___/___

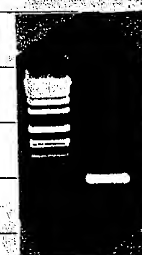
Declaration 1
Exhibit 1 Page 4
US 09/287,632

Experiment: .... TUESDAY Date

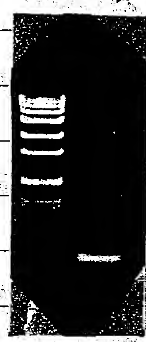
AMPLIFICATION OF Intron 2 from Pdk 1

PCR REACTION - Set up 2.

DNA (20ng)	1.0 µl	[95°C 2mins] x 1
Ampltaq BUFFER (x10)	1.0 µl	[95°C 20sec, 56°C 30 sec, 72°C 1min] x 30
$MgCl_2$ (25mM)	1.0 µl	[72°C 5 mins] x 1
P.187 { 5' PRIMER (20ng)	1.0 µl	
3' PRIMER (20ng)	1.0 µl	* Did 2 identical reactions (10 µl each)
dH_2O	3.8 µl	Ran 2.5 µl of one reaction
2.5 mM dNTP's	1.0 µl	* Pooled remainder, ø prep etc
Amplitaq Polym	0.2 µl	2 µl from 10 µl
TOTAL	10.0 µl	



767 bp Intron



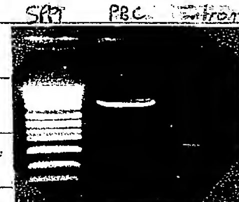
Cut rest (8 µl) in 200 µl Reaction with Hind III

LIGATION Intron 2 → PBC

Vector (PBC:H3)	: 1.0 µl	✓
INSERT (Intron 2 H3 PCR)	12.0 µl	✓
BUFFER	: 2.0 µl	✓
H_2O	: 3.0 µl	✓
LIGASE	: 1.0 µl	
ATP (5mM)	: 1.0 µl	✓
TOTAL	20.0 µl	@ 25°C

Hind III o/n

SPPI PBC Intron 2 ← Wizard cleaned



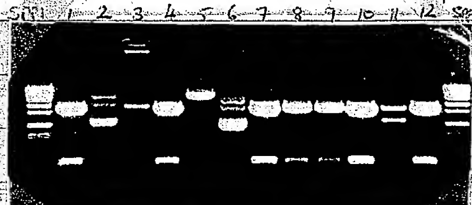
Ran 1 µl from 20 µl

Intron 2 (767bp)

chl^R B/W selection CUT WITH BglII / Pst → 577 bp OR 190 bp.

Hind III

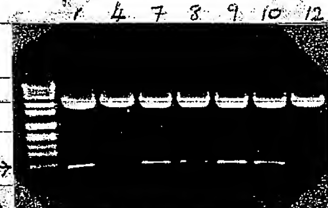
SPPI 1 2 3 4 5 6 7 8 9 10 11 12 SPPI



← 767 bp Intron 2

BglII/Pst

1 4 7 8 9 10 12



5' end → Intron 2 (~580bp)

HincII BglII 580 bp

① PBC → kpn Dra xho Sal cla H3 [INT 2] H3 RV RI Pst Sma 1, 7, 8, 9, 10

BglII

② PBC → kpn Dra xho Sal cla H3 [INT 2] H3 RV BgIII Pst Sma ... 4 + 12

HincII

Neal Smith

Signature of Researcher ....

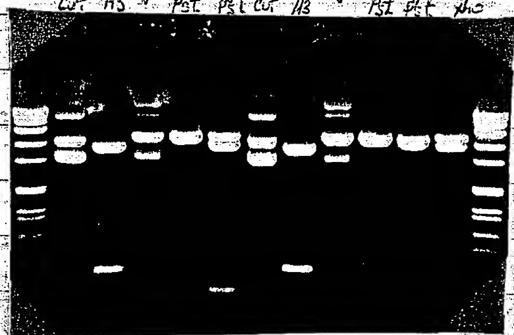
Signature of Supervisor .... HincII (blunt) Date ..../..../....

Neal Smith

Experiment: .... Date ___/___/___

INTRON 2 IN PBC

#① UN CUT, H3, BglII, BglII Pst · #④ UN CUT, H3, BglII, BglII Pst, BglII Xho



#① - In oci ① p. 188

#④ - In oci ② p. 188

NB: BglII has only partially cut

* All cuts are correct *

INTRON 2 → pNS2 (35S / PVY / H3 S4 H3 / PVY / OCS 3') Amp^R

(cut with H3)

SET UP 2 LIGATIONS ① + ②

	①	②
VECTOR (NS2-H3 A Phosph^d)	✓ 5.0 µl	✓ 1.0 µl
INSERT (INTRON 2 #1 / PBC H3)	✓ 5.0 µl	✓ 5.0 µl
BUFFER	✓ 2.0 µl	✓ 2.0 µl
H2O	✓ 6.0 µl	✓ 10.0 µl
LIGASE	✓ 1.0 µl	✓ 1.0 µl
ATP (5mM)	✓ 1.0 µl	✓ 1.0 µl
TOTAL	20.0 µl	20.0 µl

R.T ligat^n

① NS2 AP: SPP. H3

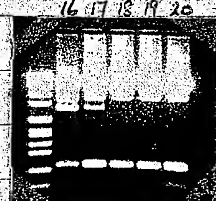
② H3: A Phos NS2^r, INTRON 2 PBC

NS2 cut looks partial

Hind III

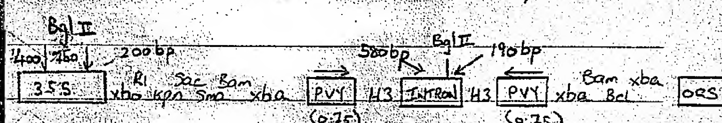
SINGLE COLONIES ① Pools x6: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

② Pools x6: 16 17 18 19 20



← INTRON 2 (770 bp)

← S4 (550 bp)



1+2 (MORE CUTS)

① UN-CUT, H3, xho, BglII, xho BglII, H3 BglII · ② UN-CUT, H3, xho, BglII, xho BglII, H3 BglII

	Sense INTRON	α-Sense Intron
✓ ② H3 → (Buffer)	770 bp (INTRON)	"
② xho →	Linearize	
✓ ③ BglII →	1.530kb (35S 3' / PVY / INT 5') + 750 from 35S	1.14 (35S 3' / PVY / INT 3') + 750
③ ✓ xho/BglII →	1.33 (PVY / INT 5') + 750 (35S 3')	950 (35S 3') + 940 (PVY / INT 3')
✓ ② H3/BglII ✓	950bp (35S 3' / PVY) / ✓ 580 (INT 5') / ✓ 190 (INT 3')	SAME

All Correct in Hind sight

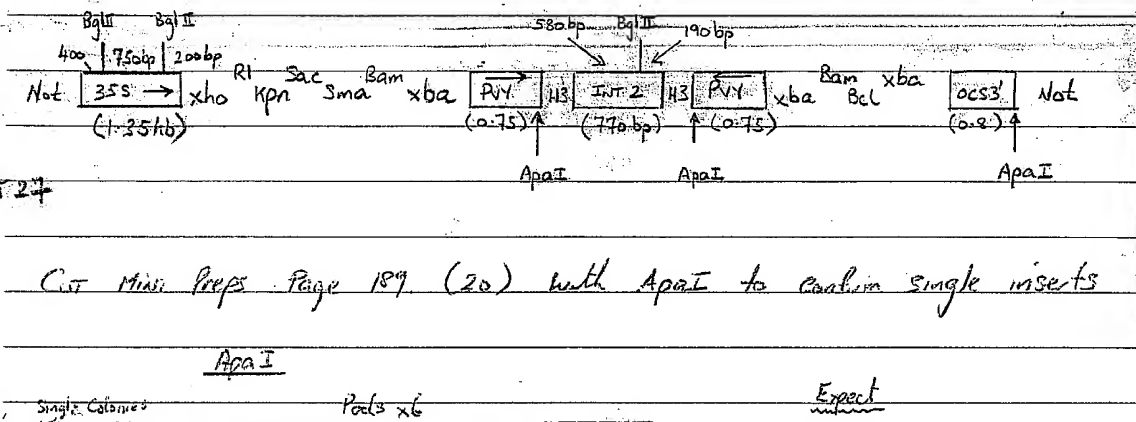
Signature of Supervisor .... Date ....

CONTINUED P. 192

Experiment: ..... THURSDAY Date ...

INTRON 2 → PNS 2 (PVY HAIRPIN) CONT' P189

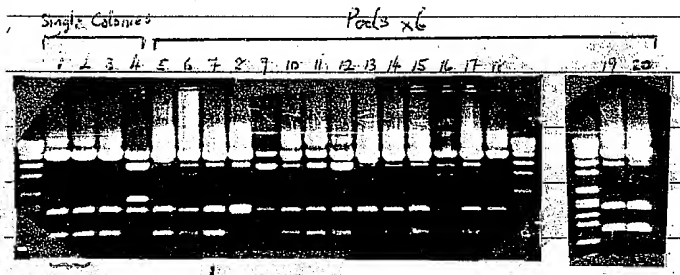
1 CONFIRMS 2 BglII SITES IN 35S



A
RB
IN ART 27

Cut Mini Preps Page 189 (20) with ApaI to confirm single inserts

ApaI



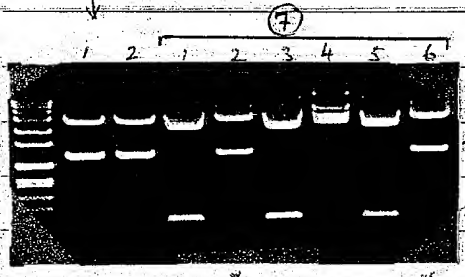
Expect

770 bp (Intron) +

~1.5 kb (PVY/ocs3') (confirms with Cuts p. 100)

✓ Appear to be many with single Intron 2 inserts

Grew up single Colonies from #7
+ cut with XbaI



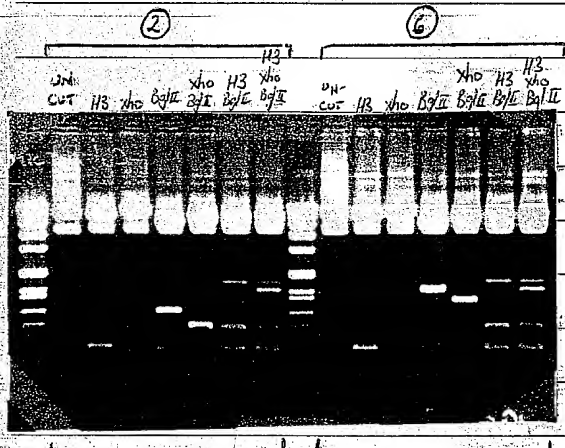
Expect

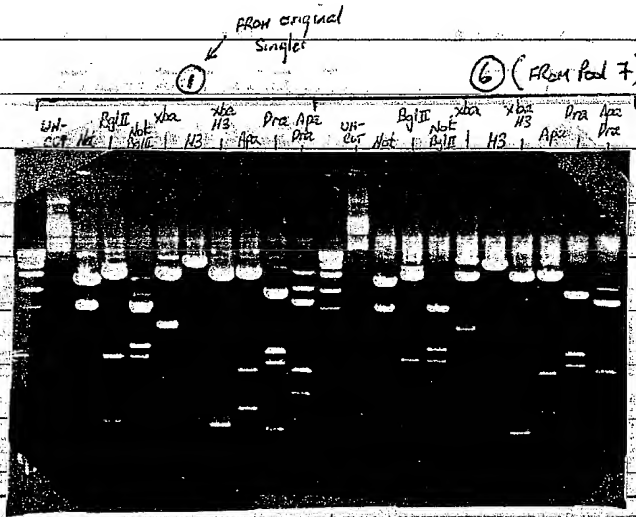
XbaI → ~2.3 kb (PVY/INT/PVY)

Again original 1+2 (p. 189)

+ 2+6 (from pool 7) appear to have intron insert

PVY deletion? due to Hairpin structure?





INTRON IN 2-SENSE ORI

INTRON IN SENSE ORI

ART 27

Signature of Researcher ..... Date .....

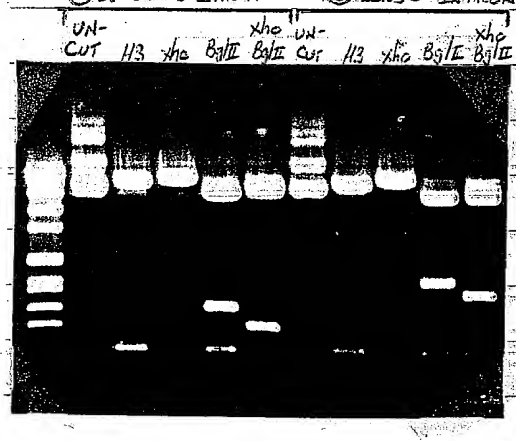
Signature of Supervisor ..... Date .....

SET UP GIAGENS OF ⑤ + ⑥ + TRI-PURIFICATIONS

Experiment:..... Monday Date.....

QIAGEN DIAGNOSTIC CUTS

② α-SENSE INTRON ⑥ SENSE INTRON



✓ All Cuts are correct.

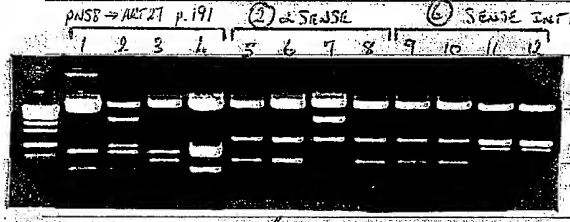
DENOTED: ② α Sense Intron as pNS 9

⑥ SENSE Intron as pNS 10

LIGATIONS. FOR ② + ⑥ → ART 27

✓ VECTOR (ART 27 p.106) 1/20	:	1.0 µl	
✓ INSERTS (② or ⑥ Not I)	:	4.0 µl	
✓ BUFFER	:	2.0 µl	② R.T O/N
✓ H_2O	:	11.0 µl	
✓ LIGASE	:	1.0 µl	B/W Spec^R.
✓ ATP (5mM)	:	1.0 µl	

BamHI cut -

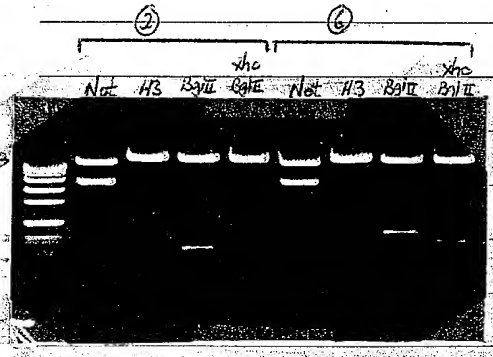


FOR ② + ⑥

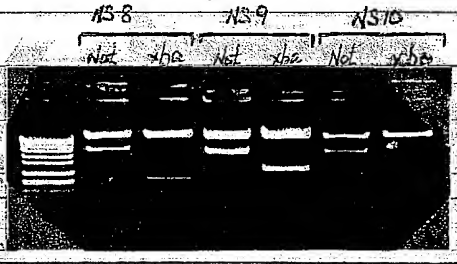
✓ 35S at RB = 1.346 (accessioned pt II) + 230kb (PVH / INT

✓ MORE CUTS TO CONFIRM

* chose # ⑧ + ⑨ FOR FURTHER CUTS



* SET UP ② + ⑥ FOR TRI-PARENTAL. → LBA 4404



Expect

NS8	Not	4.25 (INSERT)
	Xho	1.55 + 1.05 kb
NS9	Not	4.40
	Xho	~2.30
NS10	Not	4.40
	Xho	~2.30

* All Correct *

Signature of Researcher..... Date...../...../.....

Signature of Supervisor..... Date.....

Project (code) Book No: Cont'd from page

Experiment Title To make invert-Repeat GUS construct for rice transformati

①	pWHJ1adT	2μl	△ ②	pWHJadT	1μl
	10×EcoRI	5		10×buffer 4	2
	EcoRI	2.0		SmaI	1.5
	H₂O	~~38~~ 41μl.		H₂O	15.5

V: Purify ① with phenol/chloroform extr. 3 times, treat with label kit to blunt. Then purify with phenol/chloroform again followed by AP treatment.

△: Treat ② with AP.

③	pWHKKGUS	5μl	④	pWHKK-GUS	5μl
	10×buffer 3	5		10×buffer 4	5
	HincII	2		SmaI*	2
	H₂O	38		EcoRV	2
				H₂O	36

* SmaI added first.

→ Run 4.5μl of each reaction (during digesth & AP treatment).

Finally purify with phenol/chloroform.

20μl DNA in H₂O.

Ligation:

1.	DNA 1	2μl	2	DNA 1	2μl	3.	DNA 2	2.0	4	DNA 2	2.0
	10×lig	2		DNA 3	~~3.0~~ μl		10×lig	2		DNA 4.	3.0
	ATP	1		10×lig	2		ATP	1		10×lig	2
	Ligase	0.5		ATP	1		Ligase	0.5		ATP	1
	H₂O	14.5		Ligase	0.5		H₂O	14.5		Ligase	0.5
				H₂O	11.5					H₂O	11.5

4°C.

Cont'd on page

Recorded by Print Name MING-BO WANG Date

Signed

Supervisor

Initialled

Read and Understood by Print Name Date

Signed

Decl. 2 Exhibit 1 Page 2
US 09/287,632

Project (code) **Book No:** **Cont'd from page**

Experiment Title Analyse colonies of lig 2 and 4 on page 29

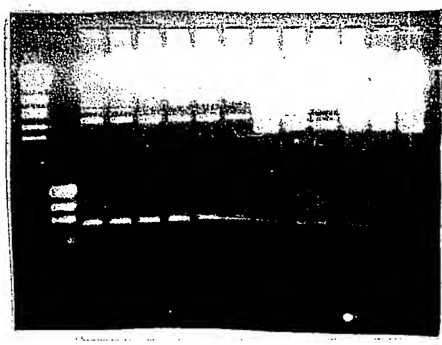
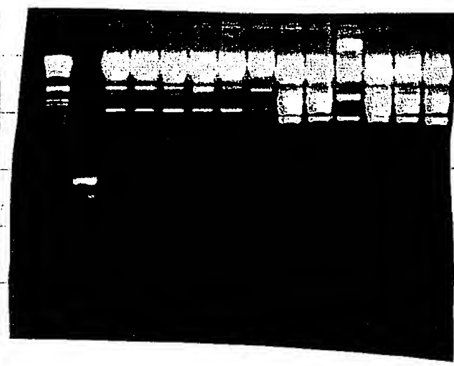
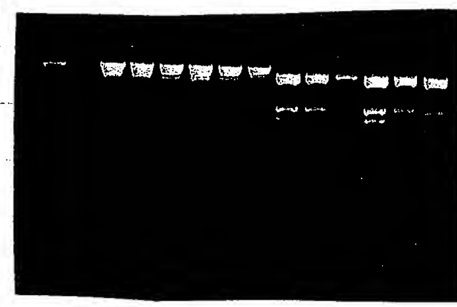
37 colonies for Lig 2.

35 " " for Lig 4.

pool thee 72 colonies into 12 tubes. 60 µl DNA in TE

Digestion:

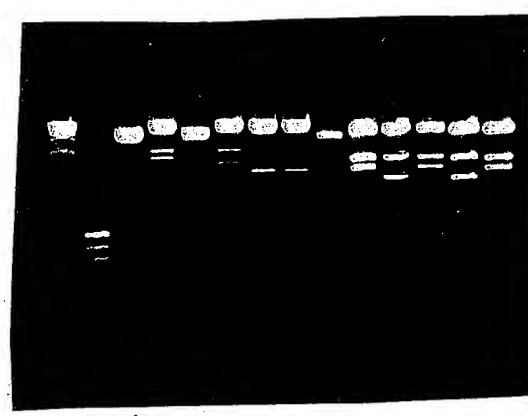
1, 2, 3, 4, 5, 6	3 µl	7, 8, 9, 10, 11, 12	
10x buffer 4	2	10x EcoRI	[illegible]
SnaBI	0.5	NcoI	0.
SacI	0.5	EcoRI	0.
H₂O	14 µl	H₂O	14

Prepare DNA from separate colonies in pool 1 and pool 7.

60 µl DNA in TE.

Digest [illegible] as above.

2 (2) : Ubi-P → 35mV GUSd → GUS5' tml' —— pMBW234

2 (4) : Ubi-P → 35mV GUSd → GUS5' tml' —— pMBW233

4 (2) 4 (4) 4 (6) Ubi-P → GUS/EcoRV GUSd → tml' —— orientation not checked

Cont'd on page pMBW

Recorded by
Print Name MING-BO WANG Date

Signed [signature]

Supervisor
Date

Initialled

Read and Understood by
Print Name Date

Signed [signature]

ject (code) **Book No:** **Cont'd from page**

xperiment Title Put pMBW232 and pMBW233 to binary pNBVec4a

1.

pMBW232	2.5 µl
10x buffer 3	5
Not I	2
H2O	40.5

2.

pMBW233	2.5
10x buffer 3	5
Not I	2
H2O	40.5

Ligations :

1.

DNA 1 ( )	1.5 µl
10x lig	2
ATP	1
Ligase	0.5
H2O	15 µl

2.

DNA ( )	1.5 µl
DNA1 ( )	3 µl
10x lig	2
ATP	0.0
Ligase	0.5
H2O	12

TO further check :

3.

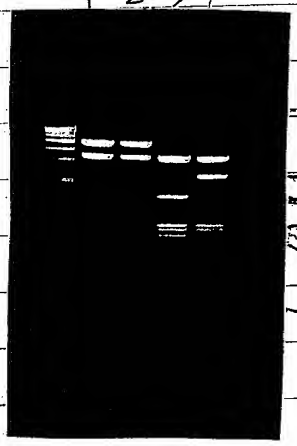
pMBW232	1 µl
10x buffer 4	2
NcoI	0.5
HindIII	0.5
H2O	16

4.

pMBW233	1 µl
10x buffer 4	2
NcoI	0.5
HindIII	0.5
H2O	16.

3.

DNA ( )	1.5
DNA2 ( )	3
10x lig	2
ATP	1
Ligase	0.5
H2O	12.



Purify 1 and 2, 20µl DNA in H2O.

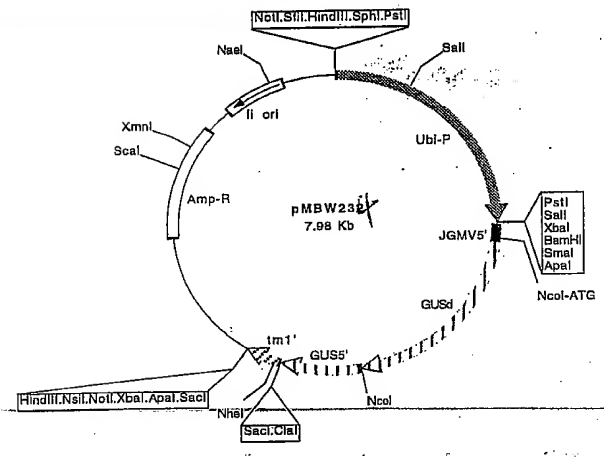
Run 2µl each together with 3 and 4.

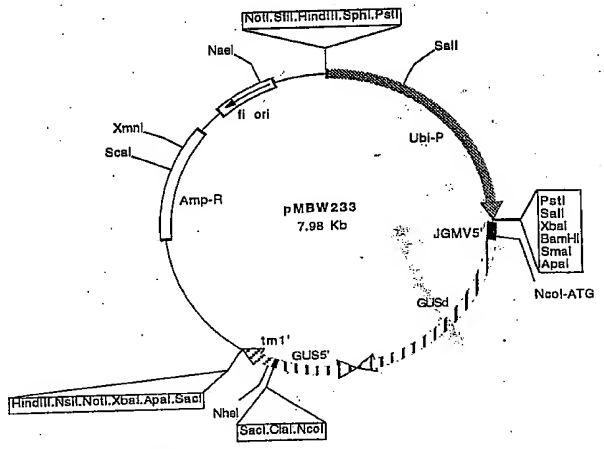
- GUSd + GUS 5' (NcoI/~~BamHI~~ HincII)
- GUSd
- ubi-P
- ubi-intron

tm1' + GUS 5' (NcoI/HincII part)

→ tm1'

* Both clones are correct.





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Project (code) Book No: Cont'd from page

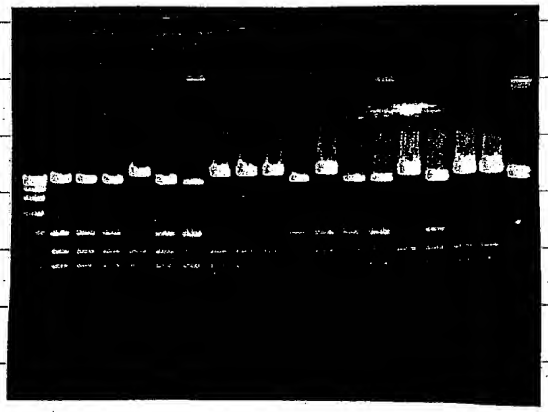
Experiment Title Analyse colonies from Lig 2 and 3 of

Bo_ve DNA is TER.

Digestion:

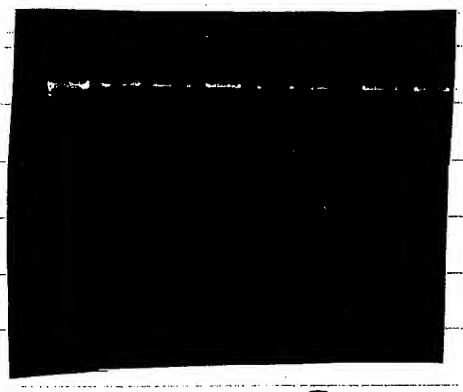
DNA	1.5ml	20 20 23 25 26	: pMBW236
10x buffer 3	2	24 27 28 29	: pMBW237
PstI	0.5	31 33 34 36	: pMBW238
H2O	16ml	35 37 38	: pMBW239

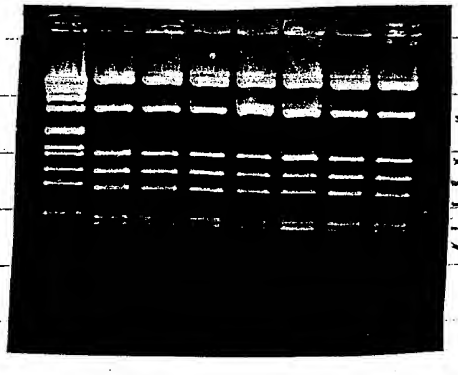
32 and 39 did not look right.



Further check.

23	24	28	32	34	35	38	1.5ml
10x buffer 3							2
NotI							0.5
~~PstI~~							
SalI							0.5
H2O							15.5





- 35S-cmsd-cmsf-tml
- ubi-p-3' gust
- LB+tml'+bar 3' half
- RB ?
- 35S-bar 5' half.
- RB or ubi-p 5' ?

28 and 38 were used for biparental mating.

Cont'd on page

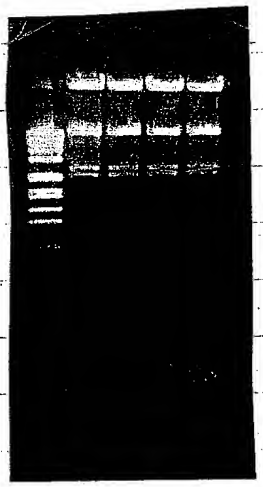
Recorded by Print Name ..... Date	Supervisor Date	Read and Understood by Print Name ..... Date .....
Signed	Initialled	Signed .....

Project (code) Book No: Cont'd from page

Experiment Title Analyse Agrobacterium transconjugants.

1, 2. AgLI = pMBW237 (28)

3, 4 : AgLI = pMBW239 (38).



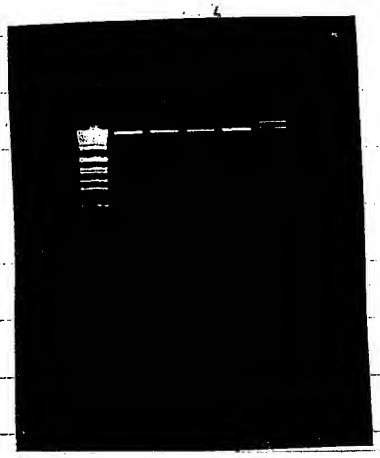
Digestion:

DNA	5µl
10X buffer 3	2
PstI	0.5
H_2O	12µl

check the JAMV vectors for KpnI.

(1) pWUJJT (No2) (2) pWUJJiCT (No10) (3) pWUJJiFT (18) (4) pWUJBiET (22) (5) pWUJBiFT (16)

10X buffer	1
KpnI	2
H_2O	0.5
	16.5



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Transgenic Research **5**, 213–218 (1996)

TECHNICAL NOTE

Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants

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Received 7 July 1995; revised 21 August 1995; accepted 24 August 1995

A set of plasmids has been constructed utilizing the promoter, 5′ untranslated exon, and first intron of the maize ubiquitin (*Ubi-1*) gene to drive expression of protein coding sequences of choice. Plasmids containing chimaeric genes for ubiquitin-luciferase (*Ubi-Luc*), ubiquitin-β-glucuronidase (*Ubi-GUS*), and ubiquitin-phosphinothricin acetyl transferase (*Ubi-bar*) have been generated, as well as a construct containing chimaeric genes for both *Ubi-GUS* and *Ubi-bar* in a single plasmid. Another construct was generated to allow cloning of protein coding sequences of choice on *Bam* HI and *Bam* HI–compatible restriction fragments downstream of the *Ubi-1* gene fragment. Because the *Ubi-1* promotor has been shown to be highly active in monocots, these constructs may be useful for generating high-level gene expression of selectable markers to facilitate efficient transformation of monocots, to drive expression of reference reporter genes in studies of gene expression, and to provide expression of biotechnologically important protein products in transgenic plants.

Keywords: gene expression; transgenic monocots; ubiquitin

Introduction

The general availability of strong promoters active in all or most cell types of monocotyledonous plants would be useful in a variety of applications in gene transfer studies with this plant group (McElroy and Brettell, 1994). Although the widely-used cauliflower mosaic virus (CaMV) 35S promoter is active in monocot cells, its relative strength is substantially less than in dicot cells, and it is inactive in some cell types, e.g. pollen (Bruce *et al.*, 1989; Christensen *et al.*, 1992; McElroy and Brettel, 1994). The maize *Adh1* promoter has also been used in monocot transformation studies (Fromm *et al.*, 1990), but its activity appears to be restricted to root and shoot meristems, endosperm, and pollen (Kyozuka *et al.*, 1991). Because of their expected involvement in fundamental processes in all cell types, the genes for rice actin (*Act-1*) (McElroy *et al.*, 1990) and maize ubiquitin (*Ubi-1*) (Christensen *et al.*, 1992) have been investigated as potentially useful alternatives to the CaMV 35S and *Adh1* sequences. Both of these monocot promoters have been shown to be significantly more active than the CaMV 35S promoter in monocot cells (Bruce *et al.*, 1989; McElroy *et al.*, 1990; Christensen *et al.*, 1992; Cornejo *et al.*, 1993; Gallo-Meagher and Irvine, 1993; McElroy and Brettell, 1994) with the *Ubi-1* promoter being somewhat stronger than the *Act-1* promoter where compared directly (Cornejo *et al.*, 1993; Gallo-Meagher and Irvine, 1993; Schledzewski and Mendel, 1994; Wilmink *et al.*, 1995).

Since our initial reports on the use of maize *Ubi-1* promoter constructs in transient (Christensen *et al.*, 1992) and stable (Toki *et al.*, 1992; Uchimiya *et al.*, 1993) cereal transformation studies, we have distributed to a large number of researchers a variety of constructs with the *Ubi-1* promoter fused to a spectrum of selectable and scorable markers. Certain of these constructs or their derivatives have been used successfully in transforming a number of different monocot species (Wilmink *et al.*, 1995), including several cereals (McElroy and Brettell, 1994) and *Lemna* (Rolfe and Tobin, 1991), with reports of transgenic plants having been generated for rice (Cornejo *et al.*, 1993), wheat (Weeks *et al.*, 1993), and

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barley (Wan and Lemaux, 1994). This report presents the structural details of the complete set of these constructs.

Materials and methods

The cloning and sequencing of the maize ubiquitin gene and its promoter have been reported previously (Christensen *et al.*, 1992). All DNA cloning and manipulations reported here were performed according to standard protocols (Ausubel *et al.*, 1989). Restriction endonuclease digestions were carried out according to manufacturers' recommendations. GeneClean (BIO 101, La Jolla, CA, USA) was used to isolate specific restriction fragments from agarose gels. Recovery of DNA fragments was quantified by comparison of ethidium bromide fluorescence of an aliquot of the fragment with known masses of DNA on agarose gels. The *Hin*dIII linker (5′ CAAGCTTG 3′) used in the construction of pAHC27 was obtained from New England Biolabs. DNA ligations and subsequent transformations into competent *Escherichia coli* strain XL1-Blue or HB101 cells and plasmid DNA preparations were carried out using standard protocols (Ausubel *et al.*, 1989). Analysis of DNA sequences was performed using the UWGCG package of programs (Fromm *et al.*, 1990) and DNA Inspector II (Textco, W. Lebanon, NH, USA).

Results

All of the constructs described here were generated by fusing the same 1992 bp *Pst*I fragment from the maize *Ubi-1* gene upstream of the relevant polylinker or marker sequence (Fig. 1). This *Ubi-1 Pst*I fragment contains 899 bp of promoter sequence, 83 bp of 5′ untranslated exon, and 1010 bp of first intron sequence, terminating through reconstitution of the *Pst*I site precisely at the G in the AG dinucleotide of the 3′ splice junction of the intron (Christensen *et al.*, 1992). The nucleotide sequences at the fusion junctions at the 3′ end of the *Ubi-1* DNA are shown for each construct in Fig. 2.

pAHC17

This plasmid is a *Ubi-1* promoter expression vector for *Bam*HI (or *Bam*HI-compatible) cloning of protein coding regions. It contains the *Ubi-1* promoter, 5′ untranslated region and intron upstream of an unique *Bam*HI site (Fig. 1). About 250 bp of nopaline synthase (NOS) 3′ untranslated sequence and polyadenylation signals are located downstream of the *Bam*HI site. The 1992 bp *Pst*I fragment of the *Ubi-1* gene had been previously cloned into the *Pst*I site of M13mp19 for sequencing (Christensen *et al.*, 1992). A *Hin*dIII-*Bam*HI fragment from the replicative form of that clone was isolated and ligated to a 3175 bp *Hin* dIII–*Bam*HI fragment of pMF6 (Goff *et al.*, 1991) containing pUC8 sequence and 250 bp of NOS 3′ polyadenylation sequence adjacent to the *Eco*RI site.

The polylinker sequence is located between the end of the *Ubi-1* intron and the *Bam*HI cloning site and between the *Bam*HI site and the NOS sequence (Fig. 2). Thus, a reporter gene cloned into the *Bam*HI site is flanked by polylinker sequence on both the 5′ and 3′ sides. *Sal*I and *Xba*I sites from the M13mp19 polylinker are upstream of the *Bam*HI site and a *Sal*I and a *Pst*I site from the pUC8 polylinker are on the 3′ side.

pAHC15 and pAHC27 (pUbi-GUS)

These plasmids contain the maize *Ubi-1* promoter, 5′ untranslated region and first intron fused to the coding region of the *E. coli uid*A gene (GUS) (Fig. 1). To produce pAHC15, *Hin*dIII–*Eco*RI fragment of pBI101.2 (Jefferson *et al.*, 1987) containing the *Hin*dIII to *Sma*I region of the pUC19 polylinker, the GUS coding sequence, and 260 bp of the nopaline synthase gene polyadenylation signal was cloned into the *Hin*dIII and *Eco*RI sites of pUC19 (pUC19-GUS-NOS). The 1992 bp *Pst*I fragment of the maize *Ubi-1* gene (Christensen *et al.*, 1992) was cloned into the *Pst*I site of the polylinker sequence upstream of the GUS coding sequence in pUC19-GUS-NOS. The construct contains the *Ubi-1* sequence in an orientation such that transcription will proceed through the ubiquitin 5′ exon, intron and the GUS coding sequence, terminating in the NOS 3′ sequence.

pAHC27 contains the same Ubi-GUS-NOS construct as pACH15 but as a *Hin*dIII fragment cloned into the *Hin*dIII site of pUC19 (Fig. 1). This construct was generated to facilitate the production of pAHC25 (see below). The *Eco*RI site at the 3′ end of the chimeric gene in pAHC15 is not unique as there is an additional *Eco*RI site in the *Ubi-1* intron. However, the *Hin*dIII site at the 5′ end of the chimaeric gene is unique. To allow the entire construct to be removed as one fragment for further subcloning, a *Hin*dIII site was introduced at the 3′ end of the chimaeric gene. This was achieved by partially digesting pAHC15 with *Eco*RI, optimizing the digestion for linear fragments. The *Eco*RI sites were filled in with dNTPs and Klenow fragment of DNA Polymerase and a *Hin*dIII linker (5′ CAAGCTTG 3′; New England Biolabs) was added. Addition of the linker also restored the *Eco*RI site. The DNA was digested with *Hin*dIII to remove excess linker and to cut at the 5′ end of the chimaeric gene. The 4.15 kb *Hin*dIII fragment containing the *Ubi-1 gus* chimaeric gene was gel-purified and subcloned into *Hin*dIII–digested pUC19. The chimaeric gene in the resultant pAHC27 is oriented such that the entire pUC19 polylinker is upstream of the *Ubi-1* promoter (Fig. 1).

pAHC18 – pUbi-LUC

This plasmid contains the *Ubi-1* promoter-5′ exon-first

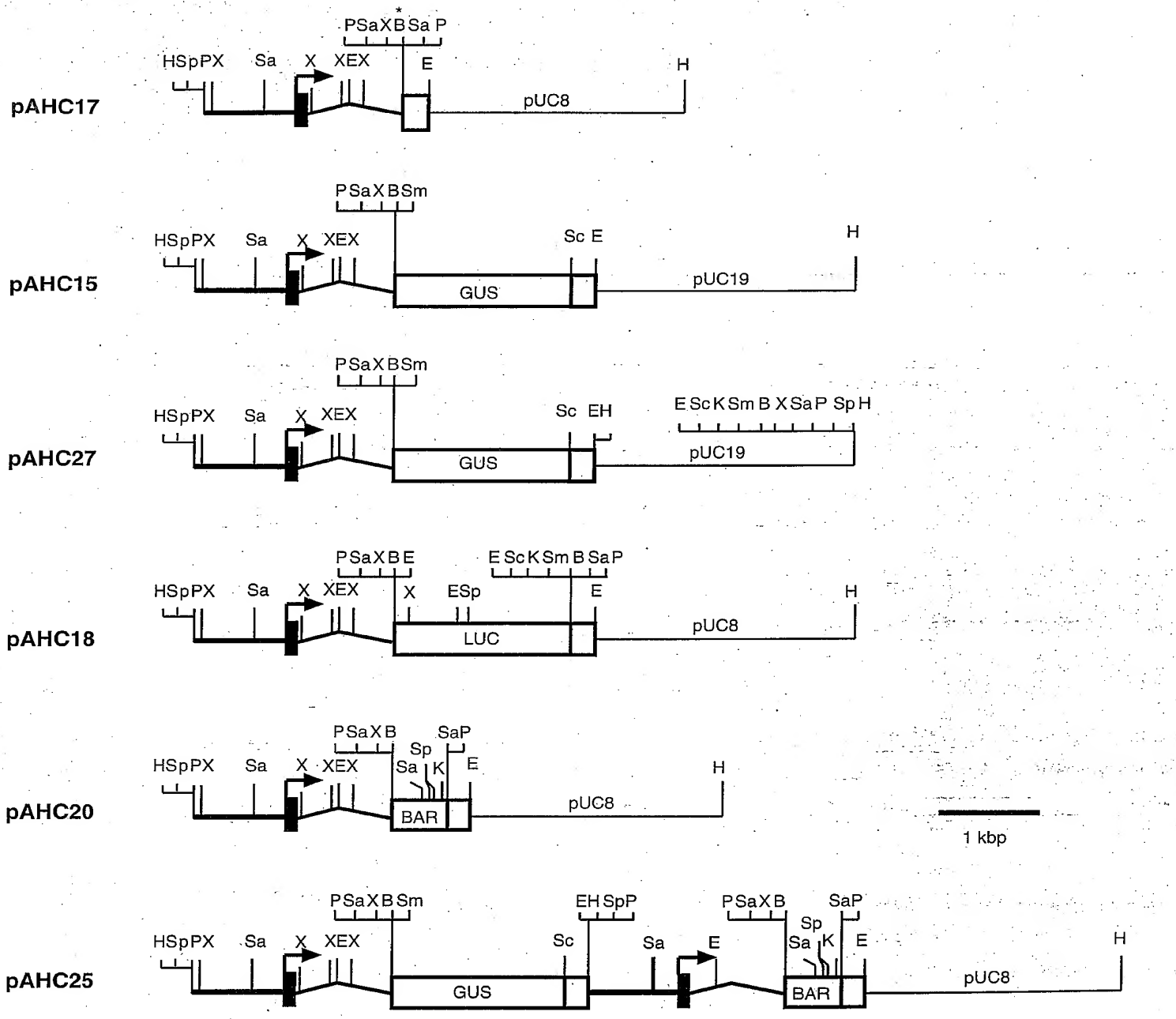

Fig. 1. Schematic diagrams of expression vectors based on maize *Ubi-1* sequences. The relative sizes of the various segments of the linearized plasmids are drawn to scale. Bold straight line, *Ubi-1* promoter sequences; filled box, *Ubi-1* exon; angled line, *Ubi-1* intron; labelled open boxes, reporter gene sequences; blank open box, nopaline synthases 3′ untranslated sequence; thin straight line, pUC8 (pAHC17, 18, 20, and 25) or pUC19 (pAHC15 and 27) sequence. Arrow at the Ubi-1 exon signifies transcription start site and direction. GUS, β-glucuronidase (Jefferson *et al.*, 1987); LUC, firefly luciferase (Ow *et al.*, 1986); BAR, phosphinothricin acetyltransferase (De Block *et al.*, 1987). Restriction sites used in construction of the chimaeric genes and in adjacent polylinker sequences are shown. The *Bam* HI site marked with an asterisk in pAHC17 is an unique site for cloning *Bam* HI or *Bam* HI-compatible fragments. (Note: The *Xba* I sites located in the Ubi-1 intron are subject to methylation interference in *dam* + *E. coli* strains. Also, although the *Eco* RI site in the *Ubi-1* intron upstream of the *gus* sequence in pAHC15 is cleaved efficiently, in both pAHC27 and pAHC25 the corresponding *Eco* RI site is cut very inefficiently). B, *Bam* HI; E, *Eco* RI; H, *Hin* dIII; K, *Kpn* I; P, *Pst* I; Sa, *Sal* I; Sc, *Sac* I; Sm, *Sma* I; Sp, *Sph* I; X, *Xba* I.

pAHC17

UBI1 Intron — **NOS 3'**

Pst I Sal I Xba I Bam HI Sal I Pst I

.....ctgcagGTCGACTCTAGAGGATCCGTCGACCTGCAG.....
.....gacgtcCAGCTGAGATCTCCTAGGCAGCTGGACGTC.....

pAHC15 / 25 / 27

UBI1 Intron — **GUS (from pBI101.2)**

Pst I Sal I Xba I Bam HI Sma I

.....ctgcagGTCGACTCTAGAGGATCCCCGGGTAGTCAGTCCCTTA*TG*.....
.....gacgtcCAGCTGAGATCTCCTAGGGGCCCATCAGTCAGGGAA*TAC*.....

pAHC18

UBI1 Intron — **LUC (from pDO432)**

Pst I Sal I Xba I Bam HI

.....ctgcagGTCGACTCTAGAGGATCCGAGCTTGGAATTCCTTTGTGTTACATTCTTGAATGTCGCTCGCAGTGACATTAGCATTCCGGTACTGTTGGTAAAA*TG*.....
.....gacgtcCAGCTGAGATCTCCTAGGCTCGAACCTTAAGGAAACACAATGTAAGAACTTACAGCGAGCGTCACTGTAATCGTAAGGCCATGACAACCATTT*TAC*.....

pAHC20 / 25

UBI1 Intron — **BAR (from pUC/BASTA)**

Pst I Sal I Xba I Bam HI

.....ctgcagGTCGACTCTAGAGGATCCATCGATTAGGAAGTAACCA*TG*.....
.....gacgtcCAGCTGAGATCTCCTAGGTAGCTAATCCTTCATTGGT*AC*.....

Fig. 2. Nucleotide sequence of the polylinker region comprising the junction between the *Ubi-1* intron and the reporter gene or NOS 3′ sequence. The *Ubi-1* intron sequence (lower case) ends with a *Pst*I site containing the 3′ splice junction. The reporter gene sequences shown downstream of the polylinker are those upstream of the respective coding sequence and end with the ATG translation start codon (italicized) shown for each.

intron fused to a luciferase (LUC) reporter coding sequence (Fig. 1). An 1892 bp *Bam*HI fragment of pDO432 (Ow *et al.*, 1986) containing 80 nucleotides of 5′ untranslated sequence, the luciferase coding region (1649 nucleotides) and 163 bp of 3′ untranslated sequence was cloned into the unique *Bam*HI site of pAHC17. This construct contains the luciferase coding sequence in the same orientation as the ubiquitin promoter.

pAHC20 – pUbi-BAR

The Ubi-BAR chimaeric gene in this plasmid provides selection of transformants resistant to Basta™ herbicide (phosphinothricin) (De Block *et al.*, 1987). The Ubi-BAR construct was formed by ligating a 570 bp *Bam*HI–*Bcl*I fragment containing the *bar* gene into the *Bam*HI site of pAHC17. The *bar* gene fragment was excised from a plasmid (pUC8/BASTA) obtained from Dr M. Fromm (Fromm *et al.*, 1990). The resultant pAHC20 plasmid has *bar* in the same orientation as the maize Ubi-1 promoter (Fig. 1). The construct contains 18 bp of sequence between the *Bam*HI site and the translation start codon of the *bar* gene (Fig. 2). The *Bcl*I site is 11 bp downstream of the TGA stop codon.

The unique *Hin*dIII site at the 5′ end of the Ubi-1 sequence makes this plasmid very adaptable. This restriction site is suitable for insertion of a second chimaeric gene, such as a scorable marker also driven by a second *Ubi-1* promoter, as detailed below for pAHC25, or for any other desired promoter–gene combination.

pAHC25 – pUbi-GUS/Ubi-BAR

pAHC25 contains both a selectable marker (*bar*) and a scorable marker (GUS), each under the transcriptional control of a separate *Ubi-1* promoter (Fig. 1). The two chimaeric genes were first assembled separately in pAHC20 and pAHC27 and then the double construct was formed. This was achieved by excising the Ubi-GUS-NOS-containing *Hin*dIII fragment from pAHC27 and subcloning it into *Hin*dIII-digested pAHC20. The resultant pAHC25 plasmid has both Ubi-BAR and Ubi-GUS chimaeric genes in the same orientation.

Discussion

The high activity of the maize *Ubi-1* promoter has now been documented in transient and/or stable transformation configurations in a number of monocot systems including rice (Bruce *et al.*, 1989; Toki *et al.*, 1992; Cornejo *et al.*, 1993; Uchimiya *et al.*, 1993; Takimoto *et al.*, 1994), wheat (Taylor *et al.*, 1993; Weeks *et al.*, 1993), barley (Wan and Lemaux, 1994), sugarcane (Gallo-Meagher *et al.*, 1993; Taylor *et al.*, 1993), maize (Christensen *et al.*, 1992; Gallo-Meagher *et al.*, 1993) *Pennisetum* (Taylor *et al.*, 1993), *Panicum* (Taylor *et al.*, 1993) and *Lemna* (Rolfe and Tobin., 1991). Whether or not the high level of expression of selectable marker genes fused to *Ubi-1* actually increases the efficiency of recovery of fertile transgenic plants relative to less active promoters like that from the CaMV 35S gene is yet to be rigorously examined (see Wan and Lemaux, 1994). However, the high level of GUS expression provided by the Ubi-GUS constructs has proven valuable in enabling rapid histochemical screening of transformants for transgene activity (Cornejo *et al.*, 1993).

The original intron present in the 5′-untranslated region of the Ubi-1 gene (Christensen *et al.*, 1992) was retained in all the constructs here because of numerous previous studies showing that introns frequently strongly enhance transgene expression in cereals (Callis *et al.*, 1988; Bruce and Quail, 1990; McElroy *et al.*, 1990; Vasil *et al.*, 1993). The influence of the *Ubi-1* intron has not been tested directly, but there is evidence that this maize sequence is spliced correctly in transgenic rice cells (Toki *et al.*, 1992).

Detailed examination of the spatial and temporal expression patterns of the *Ubi-1* promoter in transgenic plants is yet to be reported. However, initial data with a *Ubi-gus* construct indicate expression in all organs of transgenic rice consistent with a potential for targeting a wide spectrum of cells (Cornejo *et al.*, 1993; Takimoto *et al.*, 1994).

An additional potentially usefull feature of the *Ubi-1* promoter is that it is stress-inducible. Both thermal and mechanical stress have been shown to cause a strong enhancement of the *Ubi-gus* transgene activity in transformed rice (Cornejo *et al.*, 1993; Takimoto *et al.*, 1994). It is possible that this fact may result in stronger expression of selectable marker fusion genes during the early stages of transformation, where recipient cells are exposed to a variety of stresses such as high osmotic pressures, particle bombardment and growth on toxic compounds. A subsequent decrease in expression level is expected upon removal of the selective conditions so that regenerated transgenic plants would presumably not continue to express the marker at high levels when it is no longer needed. The stress-inducibility of the *Ubi-1* promoter might also be useful for driving conditional expression of genes that confer tolerance or resistance to various biotic and abiotic stresses such as pathogen attack, heat and water deficit (Takimoto *et al.*, 1994).

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Experiment: Rice transformation — Super transformation, Note

Date

Initiate calli from transgenic seeds (from T0 plants of 4R-V10-28 and 4R-V10-67).

De-husk, sterilize with ethanol for ~ 1 min, then 20% bleach for ~ 25 min. Wash 4 times with H_2O. place on NB. keep at 27°C in darkness.

: Stain the tips of shoots for GUS:

V10-28: 150 total, 32 GUS-negative

V10-67: 108 total, 25 GUS-negative.

: Cut the scutellum, and place on fresh NB medium. keep at 27°C.

: transformation:

same procedure as before. Use 1% and NB-AS to wash off 2-day old bacterium from each plate (NB-R-S). The calli were from 2 to 3 subcultures. GUS expression is good for both V10-28 and V10-67. Separate two lines for the experiments.

Constructs:

AGL2 :	pMBW223	— (1)	Ubi-tml'
" :	" 225	— (2)	Ubi-JAMV-GUSd-tml' (NO stop)
" :	" 227	— (3)	Ubi-JAMV-JAMV-GUSd-tml'
" :	" 229	— (4)	Ubi-JAMV-JAMV-iCAT-GUSd-tml'
" :	" 231	— (5)	Ubi-JAMV-JAMV-iCAT-GUSd(AS)-tml'
" :	" 237	— (6)	Ubi-JAMV-GUSd-GUS5'(AS)-tml'
" :	" 239	— (7)	Ubi-JAMV-GUSd-GUS5'(AS)-tml'

Wash the calli with H_2O — Timentin twice, blot on filter paper, then transfer to NB-Timentin150-Hygromycin50-bialaphos5. Keep at 27°C.

Signature of Researcher: Ning Wang Date: --/--/--

Signature of Supervisor:

Experiment: Date / /

[ ] Transfer all calli to NB - Timentin 150 - hygromycin 50 - bialaphos 10 mg/L.

[ ] Transfer all calli to NB - Timentin 150 - hygromycin 25 - bialaphos 10 mg/L.

[ ] Transfer resistant callus pieces to NB - Timentin 150 - hygromycin 25 - bialaphos 10 mg/L.

constructs/LUS lines	total number of callus pieces	No of bialaphos resistant callus.
1a:	pmBW223/V10-28	115	25
1b:	pmBW223/V10-67	71	36
2a:	pmBW225/V10-28	124	26
2b.	pmBW225/V10-67	63	25
3a:	pmBW227/V10-28	122	20
3b.	pmBW227/V10-67	77	22
4a:	pmBW229/V10-28	~~86~~ 113	30
4b:	pmBW229/V10-67	73	20
5a:	pmBW231/V10-28	161	22
5b.	pmBW231/V10-67	88	36
6a.	pmBW237/V10-28	125	21
6b.	pmBW237/V10-67	79	21
7a:	pmBW239/V10-28	193	28
7b.	pmBW239/V10-67	106.	23

[ ] For these lines which proliferated well and produced relatively more number of callus pieces, transfer part of the callus pieces to NB-TH50 B5, and keep part of them on the old plates. Also stain some of V10-67-derived for GUS. Look promising.

1b: lines 1, 2, 3, 5, 7, 8, 10, 11, 12, 13, 15, 17, 19, 23, 25. All GUS+ strong

2b: lines 4, 5, 10, 11, 14 = 10 not blue; 4, 14 weak; 5, 11 strong

3b. (1) (3) (5) (6) (7) (9) (8) (10) (16) (18) S - strong; ⊖ - negative; W - weak; W+S - a piece is strong, the rest is weak or ⊖.

a blue spot.

Signature of Researcher Minghua Wang Date

Signature of Supervisor M.C. Taylor Date

Decl. 2 Exhibit 3 Page 3
US 09/287,632

Experiment:..... Date ___/___/___

4b: lines: (3) (4) (5) (7) (8) (9) (12) (14) (16) (17) A blue spot

5b: lines (3) (4) (8) (9) (14) (17) (19) (23) (24) (?) A blue spot

6b: lines (2) (4) (6) very weak (8) (9) (10) (11) (13) (14) (15)

7b: lines (2) (3) (5) (6) (10) (13) (14) (15) A blue spot; A blue spot; A relatively big blue spot.
callus missing

Transfer some good callus piece onto PRT15H25BS. (*)

Transfer remaining good callus pieces into PRTH20BS or PRT15H25BS (*)

Note: (*) The medium missed glutamine.

Transfer call the calli transfered to PRT15H20BS on ___ to RTH20BS. Keep under light at 24°C.

Stain the callus piece for GUS. — see result next attached page.

(*) Some pieces also stained on ___: — Scan file plate.tif

Brightness: 150
Contrast: 178
Scaling 400%

GUS-negative or weak lines:

2a: 3, 7, 10 blue spot, 12

3a?: 2, 4 (one blue pieces one white pieces)

4a?: 5, 7

5a?: none.

6a? 1, 3.

7a 4, 8, 9.

2b: 1, 6, 12,

3b: 1, 2, 3, 12, 18.

4b: 7, 8, 11, 12, 16, 17

5b: none.

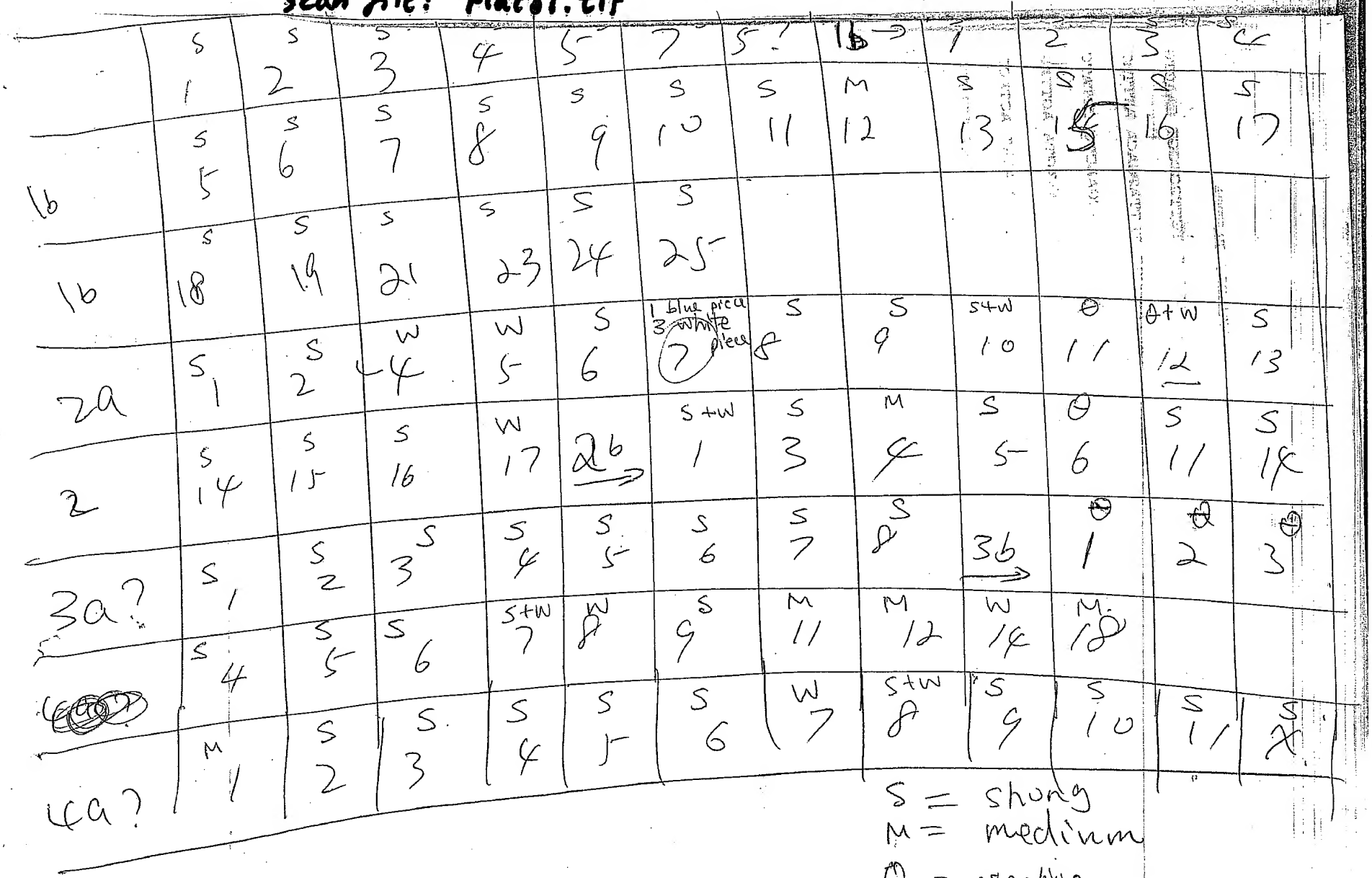
6b: 4, 6, 8, 9, 12,

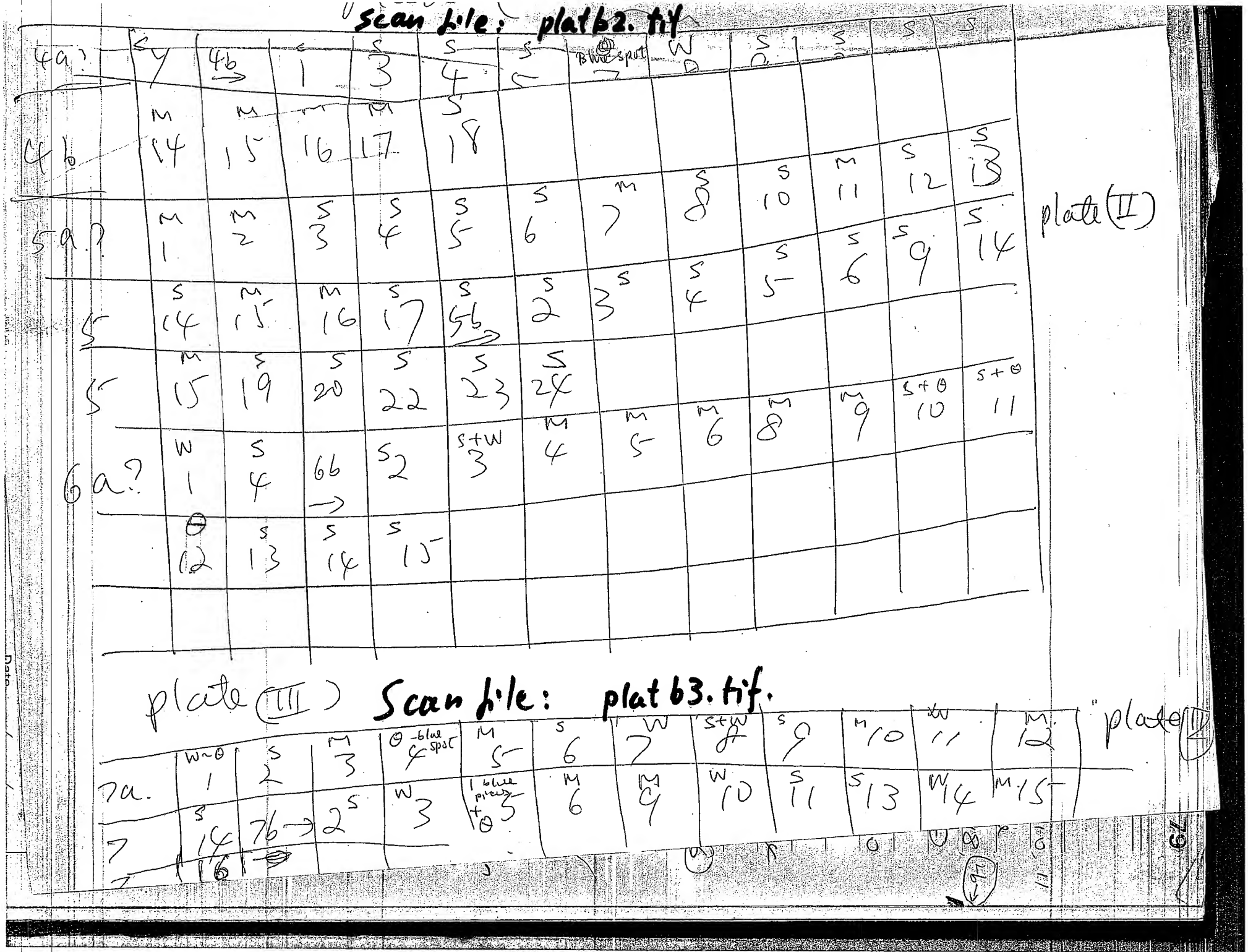
7b: 5, 3, 6

Signature of Researcher ..... Date...../...../.....

Signature of Supervisor ..... Date...../...../.....

scan file: plat01.tif





Experiment ..... Date / /

Trans fer all good calli from PRTH25BS to RTH20BS.

Stain the following for GUS:

(1a →) 1, 2, 3, 4, 5, 7, A (1b →) 1, 5, 7, 10, 11
12, 16, 17, 19, 23, 24 — All blue

(2a →) 1, (4)*, (5), (8), (10), (12), (13), (14), (15), 16, 18, (4b →)
(10) ⊖ (4), 5, 12 ⊛ ⊖ : very weak or GUS ⊖

(3a? →) 1, (2), 3, (4) 6, 7, 8 (3b? 1), (2), (3) 16, 18.

(4a? →) 1, 2, 3, 4, (5) (6) (7) 9, 10, 11, (4b →) 8, 10
11, 12, (13) (14) 15, 17

(5a? →) (1) 4, 6, 8, (9) (10), 12, 15, (16), 17, A

(6a →) (1), (3), 4, A, (B) (6b →) (4), 5, (9) (10) 11, (13) 14
15, 16

(7a →) (2), (4), (6), (7), (8), (9), (10), (11), A, (7b →) (2) (5) (6)
(10), (11), 13, 14, (15)

⊖ — very weak. GUS

⊖ — basically GUS ⊖.

☐ Stain ~~all~~ callus pieces from all lines on regeneration medium. These are all the callus lines obtained. Keep the rest for regeneration.

☐ GUS assay — see page 55, book 5.

Signature of Researcher ..... Date ... / ... / ...

Signature of Supervisor ..... Date ... / ... / ...

GUS ITP 7

Project (code) Book No: Cont'd from page

Experiment Title GUS Assay — super-transformed rice calli

Materials: choose healthy callus pieces from each line (normally a bit greener) put into 2X Eppendorf. Keep one Eppendorf at RT for protein extraction keep the other in -70°C freezer for DNA & RNA isolation. Also place a few smaller callus piece from most of the lines on NB medium for proliferation. All the callus were on regeneration medium (for a few weeks). See page 178 of book 4 for more details about the callus materials.

Protein Extraction = Follow the method on page 61 of the Thesis. Weigh the calli, add equal amount of the extraction buffer to each Eppendorf (i.e. 100μl to 100mg). Ground with the machine for ~10 sec at ~2000 rpm. Keep on ice first and then at -70°C. The next day thaw the slurry and spin twice and collect the supernatant. Store at -70°C.

Protein Quantification:

Add 800μl dH2O to a cuvette, then add 1μl of the sample, followed by 200μl BioRAD reagent solution. Mix and measure the OD at 595 nm. Make a standard curve using BSA.

Results: see the attached pages.

GUS Assay = Follow the method in the thesis: add 9μl extraction buffer, 1μl sample and 10μl reaction buffer to each well on the microtiter plate. Incubate at 37°C for ~40 min. Stop the reaction by adding 180μl 0.2M Na_2CO_3 to each well. Measure with fluroScan. The reaction solution is 4.2 mg MUG in 10 ml extraction buffer.

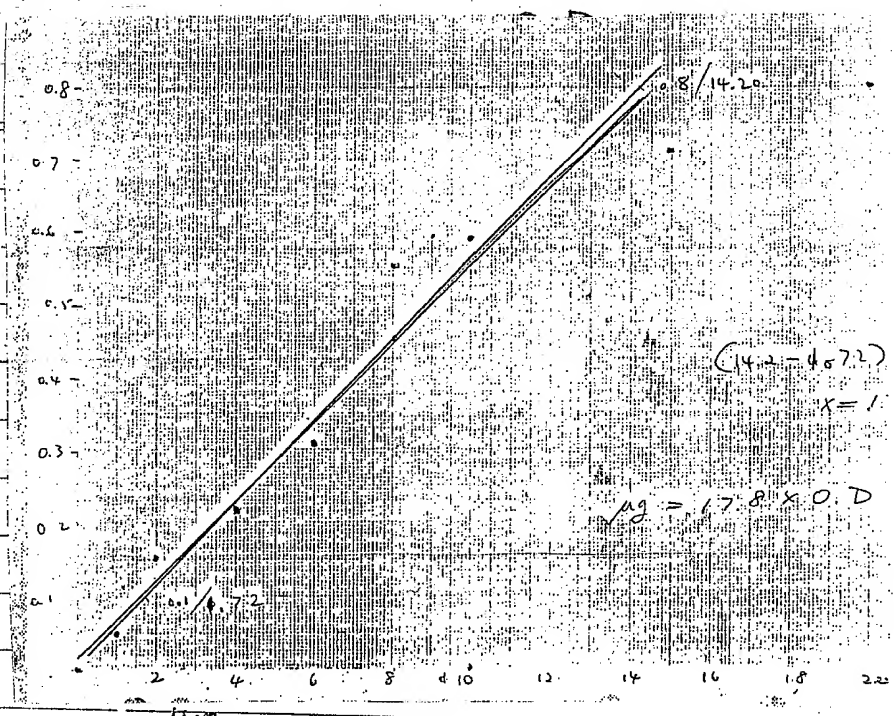
Cont'd on page

Recorded by Print Name ..... Date ..... Signed	Supervisor Date ..... Initialled	Read and Understood by Print Name ..... Date ..... Signed .....

Project (code) **Book No:** **Cont'd from page**

Experiment Title

BSA	O.D.
1 ul -	.05
2 ul -	.15
4 ul -	.22
6 ul -	.31
8 ul -	.55
10 ul -	.59
15 ul -	.71
20 ul -	.80
25 ul -	1.02



	OD	concentration (mg/ml)		OD	
NAA 1a1 -	.49	8.7	10/b12 -	.56	10.0
1a2 -	.53	9.4	11/b13 -	.54	9.6
1a3 -	.55	9.8	12/b14 -	.48	8.5
1a4 -	.53	9.4	1/b15 -	.44	7.8
1a5 -	.52	9.2	2/b16 -	.40	7.1
1a6 -	.42	7.5	3/b17 -	.47	8.4
1a7 -	.53	9.4	4/b18 -	.49	8.7
1aA -	.59	10.5	5/b19 -	.49	8.7
1aB -	.56	10.0	6/b20 -	.45	8.0
1aC -	.61	10.8	7/b20* -	.51	9.1
1b1 -	.29	5.2	8/b23 -	.37	6.6
1b2 -	.41	7.3	9/b24 -	.43	7.6
1b3 -	.21	3.7	10/b25 -	.46	8.2
2/b4 -	.36	6.4	11/2a1 -	.24	4.3
3/b5 -	.31	5.5	12/2a2 -	.30	5.3
4/b6 -	.49	8.7	1/2a3 -	.41	7.3
5/b7 -	.27	4.8	2/2a4 -	.56	10.0
6/b8 -	.46	8.2			
9/b9 -	.49	8.7			
8/b10 -	.30	5.3			
7/b11 -	.32	5.7			

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Experiment Title

3	2a5	.53	9.4	11	3a2	.25	4.4
4	2a6	.44	7.8	12	3a3	.18	3.2
5	2a7	.55	9.8	1	3a4	.18	3.2
6	2a8	.65	11.6	2	3a5	.21	3.7
7	2a9	.41	7.3	3	3a6	.25	4.4
8	2a10	.51	9.1	4	3a7	.26	4.6
9	2a11	.50	8.9	5	3a8	.19	3.4
10	2a12	.32	5.7	6	3a9	.26	4.6
11	2a13	.30	5.3	7	3aA	.28	6.8
12	2a14	.36	6.4	8	3b1	.34	6.0
1	2a15	.33	5.9	9	3b2	.55	9.8
2	2a16	.44	7.8	10	3b3	.31	5.5
3	2a17	.29	5.2	11	3b5	.32	5.7
4	2a18	.32	5.7	12	3b6	.35	6.2
5	2aA	.62	11.0	1	3b7	.42	7.5
6	2b1	.33	5.9	2	3b8	.29	5.2
7	2b2	.32	5.7	3	3b9	.23	4.1
8	2b3	.47	8.4	4	3b10	.26	4.6
9	2b4	.54	9.6	5	3b11	.31	5.5
10	2b5	.52	9.2	1	3b12	[illegible]	4.3
11	2b6	.57	10.1	7	3b14	[illegible]	5.9
12	2b7	.44	7.8	8	3b16	.62	11.0
1	2b9	.24	4.3	9	3b17	.55	9.8
2	2b10	.27	4.8	10	3b18	.29	5.2
3	2b11	.39	6.9	11	4a1	.32	5.7
4	2b12	.21	3.7	12	4a2	.22	3.9
5	2b14	.41	7.3	1	4a3	.42	7.5
6	2b16	.27	4.8	2	4a4	.31	5.5
7	2b17	.31	5.5	3	4a5	.31	5.5
8	2b19	.31	5.5	4	4a6	.32	5.7
9	2b4	.24	4.3	5	4a7	.29	5.2
10	3a1	.18	3.2	6	4a8	.36	6.4

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Experiment Title First Reading of the QCs Assay.

ate No 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	1a1 4934	1a2 4754	1a3 4783	1a4 4722	1a5 4669	1a6 4980	1a7 4311	1aA 4951	1aB 4855	1aC 4794	1b1 5286	1b2 4587
B	1b3 5325	1b4 4732	1b5 4841	1b6 4719	1b7 5070	1b8 4584	1b9 4476	1b10 4564	1b11 4648	1b12 4752	1b13 4772	1b14 4639
C	1b15 5189	1b16 4798	1b17 4949	1b18 4905	1b19 4842	1b20 4752	1b20* 4788	1b23 4850	1b24 4607	1b25 4675	2a1 4739	2a2 3086
D	2a3 5966	2a4 172.9	2a5 226.6	2a6 3426	2a7 1084	2a8 4749	2a9 4731	2a10 4931	2a11 3444	2a12 4788	2a13 4797	2a14 4518
E	2a15 5164	2a16 5778	2a17 110.1	2a18 4969	2a19 4266	2b1 4918	2b2 4909	2b3 4980	2b4 1616	2b5 4465	2b6 1574	2b7 153.8
F	2b9 733.5	2b10 4999	2b11 4798	2b12 4830	2b14 4757	2b16 4697	2b17 4721	2b19 528.4	2bA 329.7	3a1 4648	3a2 4594	3a3 4475
G	3a4 401.4	3a5 4701	3a6 4679	3a7 4523	3a8 4738	3a9 4331	3aA 4605	3b1 61.79	3b2 87.64	3b3 15.21	3b5 4473	3b6 6230
H	3b7 4590	3b8 3810	3b9 4626	3b10 4829	3b11 4571	3b12 283.0	3b14 127.8	3b16 4466	3b17 4536	3b18 4607	4a1 4418	4a2 4368

ate No 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	4a3 4874	4a4 4613	4a5 321.4	4a6 4979	4a7 1092	4a8 4239	4a9 4974	4a10 4697	4a11 4735	4a12 4851	4aA 4814	4aB 4665
B	4aC 4748	4aD 4826	4aX 4854	4aY 4866	4aZ 4840	4b1 4849	4b3 4805	4b4 4643	4b5 4798	4b6 215.8	4b7 4713	4b8 4551
C	4b9 4658	4b10 4793	4b11 318.2	4b12 4904	4b13 112.1	4b14 108.6	4b15 4755	4b16 360.3	4b17 4755	4b18 4684	5a1 396.2	5a2 1074
D	5a3 4906	5a4 4927	5a5 5005	5a6 4957	5a7 4946	5a8 4798	5a9 4025	5a10 154.8	5a11+12 4730	5a12 4892	5a13 4837	5a14 4750
E	5a15 4552	5a16 280.0	5a17 3985	5aA 4817	5b1 1708	5b2 4693	5b3 4676	5b4 4448	5b5 372.3	5b6 4608	5b8 4787	5b9 5037
F	5b10 327.8	5b12 4745	5b13 4742	5b14 4795	5b15 4727	5b16 4766	5b17 4809	5b19 1358	5b20 2719	5b22 4608	5b23 4736	5b24 4522
G	5b26 4584	5b27 3115	5b29 4775	5b30 256.5	6a1 967.9	6a3 501.5	6a4 4692	6aA 4691	6aB 682.8	6aC 73.21	6b1 4566	6b2 4557
H	6b3 650.4	6b4 4557	6b5 4638	6b6 163.6	6b8 415.2	6b9 197.6	6b10 4618	6b11 446.7	6b13 4576	6b14 4638	6b15 4407	6b16 221.5

TRANSF. 4:

te No 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	7a1 273.2	7a9+10 283.9	[redacted]	7b2 240.4	7b13 23.20	0.094	0.094	0.204	0.408	0.173	0.141	0.377
B	7a2 88.46	7a10 116.3	[redacted]	7b5 4879	7b14 218.1	0.173	0.188	0.566	0.157	0.157	0.188	0.110
C	7a3 147.5	7a11 97.77	[redacted]	7b3 46.28	7b15 134.9	0.188	0.204	0.283	0.833	0.314	0.518	0.126
D	7a4 46.48	7a12 61.26	[redacted]	7b5 121.1	7b16 78.18	0.220	0.267	0.220	0.314	0.236	0.440	0.534
E	7a5 384.9	7a14 4840	[redacted]	7b6 207.5	control 14.00	0.157	0.314	0.550	0.283	0.393	0.141	0.141
F	7a6 410.4	7aA 5031	[redacted]	7b7 128.8	control 12.35	0.126	0.094	0.283	0.408	0.660	3.236	0.188
G	7a7 999.4	7a13 36.74	[redacted]	7b10 31.40	[illegible] 9.033	0.157	0.314	0.503	0.440	0.110	0.094	0.126
H	7a8 5257	7aC 32.17	[redacted]	7b11 153.0	buffer 9.001	0.173	0.188	0.236	0.110	0.408	0.157	0.220

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Experime

4a9- .27 7 4.8
4a10- .36 8 6.4
4a11- .42 9 7.5
4a12- .25 10 4.45
4aA- .53 11 9.43
4aB- .59 12 10.5
4aC- .56 1 10.0
4aD- .50 2 8.9
4aX- .183 3.2
4aY- .184 3.2
4aZ- .22 5 3.9
4b1- .36 6 6.4
4b3- .47 7 8.4
4b4- .30 8 5.3
4b5- .49 9 8.7
4b6- .22 10 3.9
4b7- .40 11 7.1
4b8- .36 12 6.4
4b9- .36 1 6.4
4b10- .38 2 6.7
4b11- .29 3 5.2
4b12- .26 4 4.6
4b13- .25 5 4.4
4b14- .27 6 4.8
4b15- .46 7 8.2
4b16- .42 8 7.5
4b17- .48 9 8.5
4b18- .39 10 6.9
5a1- .40 11 7.1
5a2- .41 12 7.3
5a3- .36 1 6.4
5a4- .38 2 6.8
3 5a5- .37 6.6
4 5a6- .32 5.7
5 5a7- .29 5.2
6 5a8- .28 5.0
7 5a9- .36 6.4
8 5a10- .18 3.2
9 5a11+12- .21 3.7
10 5a12- .22 3.9
11 5a13- .25 4.4
12 5a14- .21 3.7
1 5a15- .17 3.0
2 5a16- .27 4.8
3 5a17- .32 5.7
4 5aA- .25 4.4
5 5b1- .58 10.3
6 5b2- .35 6.2
7 5b3- .37 6.6
8 5b4- .43 7.6
9 5b5- .32 5.7
10 5b6- .32 5.7
11 5b8- .52 9.2
12 5b9- .37 6.6
1 5b10- .17 3.0
2 5b12- .20 3.6
3 5b13- .13 2.3
4 5b14- .30 5.3
5 5b15- .39 6.9
6 5b16- .21 3.7
7 5b17- .20 3.6
8 5b19- .44 7.8
9 5b20- .30 5.3
10 5b22- .16 2.8

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5b23	.24	11	4.3		
5b24	.31	12	5.5		
5b26	.18	1	3.2		
5b27	.25	2	4.4		
5b29	.22	3	3.9		
5b30	.16	4	2.8		
6a1	.32	5	5.7		
6a3	.27	6	4.8		
6a4	.36	7	6.4		
6aA	.32	8	5.7		
6aB	.34	9	6.0		
6aC	.33	10	5.9		
6b1	.34	11	6.0		
6b2	.30	12	5.3		
6b3	.25	1	4.4		
6b4	.24	2	4.3		
6b5	.39	3	6.9		
6b6	.26	4	4.6		
6b8	.31	5	5.5		
6b9	.34	6	6.0		
6b10	.43	7	7.6		
6b11	.32	8	5.7		
6b13	.28	9	5.0		
6b14	.52	10	9.2		
6b15	.45	11	8.0		
6b16	.31	12	5.5		
7a1	.29	1	5.2		
7a2	.24	2	4.3		
7a3	.26	3	4.6		
7a4	.26	4	4.6		
7a5	.24	5	4.3		
7a6	.27	6	4.8		
7a7	.20	7	3.6		
7a8	.26	8	4.6		
7a9+10	.23	9	4.1		
7a10	.20	10	3.6		
7a11	.27	C	4.8		
7a12	.23	D	4.1		
7a14	.35	E	6.2		
7aA	.24	F	4.3		
7aB	.23	G	4.1		
7aC	.16	H	2.8		
7b2	.35	A	6.2		
7b3+5	.39	B	6.9		
7b3	.21	C	3.7		
7b5	.35	D	6.2		
7b6	.34	E	6.0		
7b7	.26	F	4.6	7aA	
7b10	.22	G	3.9		
7b11	.39	H	6.9		
7b13	.34	A	6.0		
7b14	.34	B	6.0		
7b15	.25	C	4.4		
7b16	.30	D	5.3		
Controls N°1	.44	E	7.8		
Controls N°2	.55	F	9.8		
G extraction					
H extraction					

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plate 1
second Reading

	1	2	3	4	5	6	7	8	9	10	11	12
A	4790	4695	4711	4677	4598	4634	4143	4650	4539	4596	4454	4386
B	4672	4550	4523	4702	4530	4503	4405	4514	4613	4647	4730	4465
C	4996	4710	4876	4862	4768	4693	4725	4790	4566	4631	4695	3041
D	4856	161.5	223.3	3355	994.1	4653	4648	4642	3421	4651	4668	4444
E	4832	4712	103.6	4771	4071	4670	4593	4610	1610	4439	1566	152.1
F	708.2	4645	4641	4823	4577	4545	4638	523.4	326.8	4532	4404	4402
G	389.6	4530	4598	4364	4461	4271	4315	62.14	87.80	15.52	4395	5851
H	4639	3851	4559	4585	4551	280.1	123.1	4429	4459	4453	4361	4320

TRANSF. 4:

plate 2
second reading

	1	2	3	4	5	6	7	8	9	10	11	12
A	4729	4525	314.7	4914	1075	4125	4789	4619	4645	4587	4693	4614
B	4614	4771	4775	4793	4766	4766	4709	4565	4711	212.7	4628	4497
C	4606	4743	314.5	4835	110.6	108.0	4651	355.5	4667	4607	390.5	1060
D	4725	4867	4752	4870	4861	4717	3929	152.7	4562	4698	4613	4616
E	4473	277.1	3912	4771	1696	4596	4572	4311	366.4	4429	4501	4466
F	325.9	4678	4684	4732	4658	4704	4650	1340	2678	4486	4590	4438
G	4539	3086	4705	256.5	959.3	494.8	4577	4596	673.9	72.50	4473	4497
H	621.2	4503	4578	162.2	406.1	197.1	4532	438.9	4499	4525	4304	216.6

plate 3
Second Reading

	1	2	3	4	5	6	7	8	9	10	11	12
A	280.6	293.8	8.788	245.8	23.66	0.084						
B	90.59	119.1	9.258	4959	222.9	0.084						
C	149.7	99.73	9.225	47.30	137.9	0.174						
D	44.14	61.30	8.242	124.0	80.24	0.101						
E	382.3	4819	9.158	211.5	14.74	0.185						
F	409.2	4990	8.947	130.5	17.27	0.168						
G	961.6	36.46	16.82	31.88	9.377	0.084						
H	5027	31.89	8.788	154.4	9.024	0.154						

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Experiment Title

plate 1
Second Reading
2 x dilution

	1	2	3	4	5	6	7	8	9	10	11	12
A	2836	2682	2873	2792	2805	2762	2469	2803	2718	2699	2637	2555
B	2691	2595	2719	2872	2785	2691	2680	2713	2812	2733	2890	2628
C	2973	2779	3001	2982	2980	2892	2944	2942	2776	2770	2845	1670
D	2857	72.48	107.9	1845	486.0	2801	2888	2834	1930	2785	2885	2669
E	2839	2838	48.92	2896	2357	2865	2791	2790	831.8	2661	808.5	87.91
F	328.4	2713	2826	3024	2797	2807	2929	264.5	166.1	2817	2740	2680
G	174.1	2624	2841	2620	2652	2579	2559	30.00	60.88	53.34	2730	4092
H	2684	2110	2839	2758	2716	134.0	59.37	2696	2700	2705	2628	2594

plate 2
second reading
2x dilution

	1	2	3	4	5	6	7	8	9	10	11	12
A	2797	2643	175.8	2976	543.5	2379	2906	2749	2796	2752	2820	2709
B	2682	2837	2883	2916	2885	2900	2842	2690	2868	124.1	2785	2665
C	2691	2833	178.5	2902	76.92	76.45	2800	189.8	2793	2764	207.0	522.1
D	2752	2927	2935	2988	6607	2870	2282	94.69	2723	2861	2807	2777
E	2552	152.4	2268	2978	837.9	2714	2710	2495	187.4	2635	2717	2594
F	174.4	2896	2893	2870	2820	2888	2836	678.0	1426	2705	2809	2657
G	2641	1677	2855	144.3	481.9	257.3	2757	2779	340.6	58.74	2695	2716
H	310.4	2684	2750	101.6	213.7	116.2	2728	231.0	2689	2731	2557	124.3

plate 3
Second Reading
2x dilution

	1	2	3	4	5	6	7	8	9	10	11	12
A	152.8	160.8	[illegible]	140.6	38.10							
B	68.14	82.42	[illegible]	2969	131.9							
C	94.68	73.50	[illegible]	49.31	92.04							
D	47.13	56.39	[illegible]	33.82	66.42							
E	197.5	2791	[illegible]	125.9	34.74							
F	211.9	2972	[illegible]	89.95	36.26							
G	459.2	44.20	[illegible]	43.16	32.10							
H	2859	41.82	[illegible]	100.4	31.91							

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Calibrated CUS Assay Reading

Note: Figures from the second reading were used. For large figu use the readings from 2x dilution. For small ones, use th reading without dilution. The reading were then divided b the calculated concentration of each sample ← Final data

Sample	calibrated reading	Sample	Calibrated Reading
1a1	650	1615	762
1a2	571	1616	783
1a3	586	1617	714
1a4	594	1618	686
1a5	680	1619	685
1a6	736	1620	723
1a7	525	1620*	647
1aA	534	1623	891
1aB	544	1624	730
1aC	500	1625	676
1b1	1014	2a1	1323
1b2	700	2a2	630
1b3	1454	2a3	783
1b4	811	2a4	16.2
1b5	989	2a5	23.8
1b6	660	2a6	473
1b7	1160	2a7	99.2
1b8	656	2a8	483
1b9	616	2a9	791
1b10	1023	2a10	623
1b11	987	2a11	433
1b12	547	2a12	977
1b13	602	2a13	1088
1b14	678	2a14	834

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Experiment Title

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Sample	Calibrated Reading	Sample	Calibrated Reading
2a15	962	3a4	121.
2a16	728	3a5	1418
2a17	79.9 -	3a6	1291
2a18	1016	3a7	1139
2a19	428	3a8	1560
261	971	3a9	1121
262	979	3a10	752
263	664	361	10.4 -
264	168	362	8.96 -
265	578	363	2.82 -
266	155	365	958
267	19.5 -	366	1320
269	164	367	716
2610	1130	368	811
2611	819	369	1384
2612	1634	3610	1199
2614	766	3611	988
2616	1169	3612	65.1 -
2617	1065	3614	20.9 -
2619	95.2 -	3616	490
2610	76.0 -	3617	551
3a1	1760	3618	1040
3a2	1245	491	922
3a3	1675	492	1330

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Recorded by	Supervisor	Read and Understood by
Print Name ..... Date	Date	Print Name ..... Date .....
Signed	Initialled	Signed .....

Sample	Calibrated Reading	Sample	Calibrated Reading
4a3	745	469	841
4a4	961	4610	846
4a5	57.2	4611	60.5
4a6	1044	4612	1261
4a7	207	4613	25.1
4a8	743	4614	22.5
4a9	1210	4615	683.
4a10	859	4616	47.4
4a11	746	4617	657
4a12	123	4618	801
4aA	598	5a1	55
4aB	516	5a2	145
4aC	536	5a3	860
4aD	637	5a4	861
4aX	1801	5a5	889
4aY	1822	5a6	1048
4aZ	1479	5a7	2541
461	906	5a8	1148
463	677	5a9	713
464	1015	5a10	477
465	659	5a11+12	1472
466	54.5	5a12	1467
467	784	5a13	1275
468	833	5a14	1501

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Project (code) Book No: Cont'd from page

Experiment Title

Decl. 2 Exhibit 4 Page 12
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Sample	Calibrated Reading	Sample	Calibrated Reading
5a15	1701	5626	1650
5a16	57.7	5627	762
5a17	795	5629	1464
5aA	1353	5630	91.6
561	165	6a1	168.3
562	875	6a3	103.1
563	821	6a4	861.
564	656	6aA	975
565	643	6aB	112
566	924	6aC	12.3 ✓
568	590	661	898
569	786.	662	1024
5610	109	663	141.
5612	1609	664	1248
5613	2515	665	797
5614	1083	666	35.3 ✓
5615	817	668	77.8
5616	1561	669	32.8 ✓
5617	1575	6610	718
5619	172	6611	77.0
5620	538	6613	1076
5622	1932	6614	594
5623	1306	6615	639
5624	966	6616	39.3

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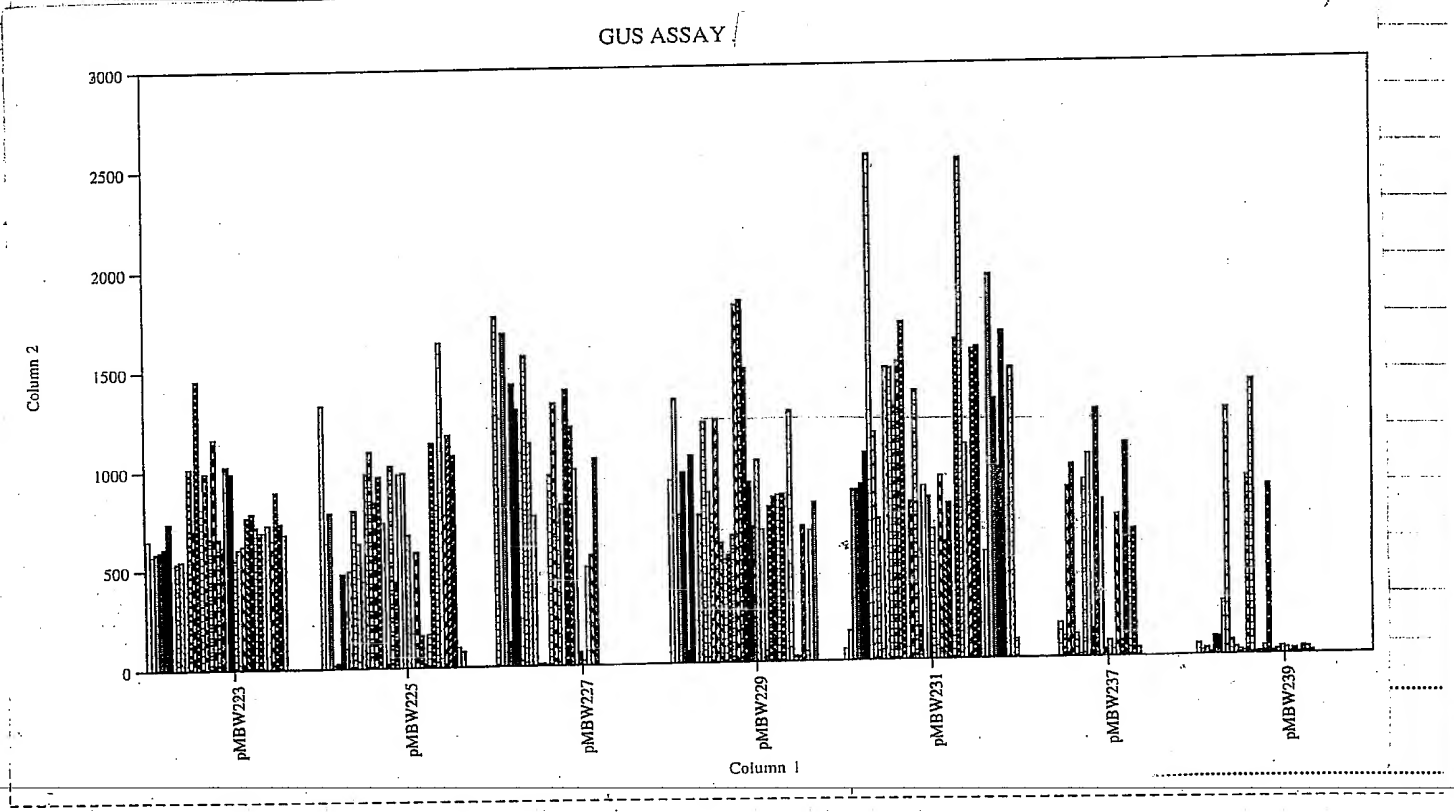
Recorded by	Supervisor	Read and Understood by
Print Name ..... Date	Date	Print Name ..... Date .....
Signed	Initialled	Signed .....

Sample	Calibrated Reading	Sample	Calibrated Reading
7a1	540	762	39.6
7a2	21.1	7635	860
7a3	32.5	763	12.8
7a4	9.60	765	20.0
7a5	88.9	766	35-2
7a6	85.2	767	28-4
7a7	2671	7610	8-17
7a8	1243	7611	22-4
7a9+10	76.6	7613	3-94
7a10	33.1	7614	37-2
7a11	20.8	7615	31-3
7a12	15.0	7616	15-1
7a14	900.8	control	1.89
7aA	1382	control2	1.76
7aB	8.90	exh. buff.	
7aC	11.7	exh. buff.	



Project (code) Book No: Cont'd from page

Experiment Title Repeat the AUS Assay for supertransformed Vice cell this time dilute the exhact to 1 mg/ml protein, use 1.5 ul for assay. Measure kinetic value rather them end point

86.

NSF. 2:
NSF. 3:
NSF. 4: plate No.1

	1	2	3	4	5	6	7	8	9	10	11	12
A	1a1 121.0	1a2 45.58	1a3 99.28	1a4 26.17	1a5 92.21	1a6 108.8	1a7 6.432	1a8 90.80	1a9 98.24	1a10 93.76	1b1 318.8	1b2 109.5
B	1b3 30.35	1b4 40.04	1b5 [illegible]	1b6 23.05	1b7 [illegible]	1b8 118.5	1b9 11.27	1b10 110.6	1b11 19.29	1b12 121.0	1c9 115.1	1c10 77.10
C	1c11 106.1	1c12 73.12	29.97	1c15 50.11	1c19 80.34	1c20 117.8	1c26 139.1	1c27 67.52	1c24 92.32	1c25 27.97	2a1 97.45	2a2 6.637
D	2a3 71.60	2a4 0.224	2a5 0.321	2a6 5.290	2a7 0.946	2a8 32.44	2a9 128.8	2a10 31.28	2a11 5.023	2a12 40.27	2a13 36.40	2a14 53.24
E	2a15 29.97	2a16 89.06	2a17 0.256	2a18 68.23	2a19 5.841	2b1 93.43	2b2 73.19	2b3 128.1	2b4 1.506	203.8 140.6	2b6 1.275	2b7 0.272
F	2b9 0.209	2b10 42.05	2b11 117.5	118.9	2b14 21.44	155.0	190.9	0.773	0.146	3a1 38.43	3a2 64.16	149.2
G	3a4 0.955	3a5 68.32	2a6 105.6	3a7 136.6	3a8 140.4	3a9 62.38	3a14 17.79	3b1 0.199	3a2 0.197	3a3 0.157	3b5 128.0	3b6 130.3
H	255.3 196.2	3b9 12.43	3b10 140.0	3b11 90.13	207.2 157.4	3b12 0.518	3b14 0.231	3b16 116.1	3b17 43.18	3b18 31.06	4a1 38.88	4a2 115.5

NSF. 2:
NSF. 3:
NSF. 4: plate No.2

	1	2	3	4	5	6	7	8	9	10	11	12
A	4a3 133.0	4a4 98.46	4a5 0.502	4a6 39.35	4a7 1.545	4a8 10.36	4a9 111.6	4a10 14.42	4a11 88.06	4a12 52.28	4a13 30.26	4a14 107.5
B	4a15 26.75	4a16 105.1	4a17 135.1	4a18 95.04	4a19 71.15	4b1 31.82	4b3 58.08	4b4 56.32	4b5 44.62	4b6 0.454	4b7 17.32	4b8 73.20
C	4b9 20.32	4b10 107.4	4b11 0.453	4b12 87.81	4b13 0.299	4b14 0.206	4b15 12.47	4b16 0.213	4b17 11.15	4b18 19.22	5a1 0.290	5a2 0.572
D	5a3 37.20	5a4 53.94	5a5 105.5	5a6 56.73	5a7 60.36	5a8 71.12	5a9 13.17	5a10 0.424	5a11 26.98	5a12 115.5	5a13 149.7	5a14 66.75
E	5a15 145.8	5a16 0.534	5a17 9.400	5a18 35.33	5b1 1.395	5b2 152.4	5b3 67.42	5b4 12.11	5b5 0.668	5b6 23.34	5b8 76.10	5b9 130.1
F	5b10 0.845	5b12 66.12	136.9	5b14 67.92	5b15 50.32	170.3	5b17 108.9	5b19 1.241	5b20 4.092	169.6	5b23 76.88	5b24 22.08
G	5b26 91.60	5b27 7.885	5b29 69.76	5b30 0.822	6a1 1.440	6a3 0.721	6a4 62.39	6a14 109.3	6a8 1.154	6a6 0.214	6b1 21.23	6b2 129.3
H	6b3 1.181	6b4 25.57	6b5 87.19	6b6 0.407	6b8 0.537	6b9 0.433	6b10 19.23	6b11 0.785	6b13 38.79	6b14 27.84	6b15 13.67	6b16 0.370

NSF. 2:
NSF. 3:
NSF. 4: plate No3.

	1	2	3	4
A	7a1 0.565	7a4 0.717	7b2 0.472	7b13 0.172
B	7a2 0.316	7a10 0.398	21.49	7b14 0.436
C	7a5 0.351	7a11 0.315	7b3 0.256	7b15 0.398
D	7a9 0.210	7a12 0.270	7b5 0.296	7b16 0.242
E	7a5 0.701	7a14 16.78	7b8 0.452	Control 0.167
F	7a6 0.733	7a15 67.28	7b7 0.422	Control 0.157
G	7a7 2.103	7a8 0.217	7b10 0.196	0.165
H	7a8 119.8	7a12 0.208	7b11 0.294	0.164

24/28 (86%)

F. 4: Further 5x dilution of a few

	1	2	3	4	5	6	7
A	1b1 82.22	3b7 51.06					
B	1b5 81.99	3b11 41.45					
C	1b7 71.57	5b13 53.15					
D	1b20 52.31	5b16 57.41					
E	2b5 40.75	5b22 68.18					
F	2b17 58.59	0.150					
G	3a3 55.23	0.140					
H	3b6 42.75	0.151					

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Proje
Exp

	1	2	3	4	5	6	7	8	9	10	11	12
A	1a1		1a3		1a5	1a6		1aA	1aB	1aC	1b1	1b2
B			165		167	168		1b10		1b12	1b13	1b14
C	1b15	1b16			1b19	1b20	1b20*	1b23	1b24		2a1	
D	2a3						2a9					2a14
E		2a16		2a18		2b1	2b2	2b3		2b5		
F			2b11	2b12		2b16	2b17				3a2	3aB
G		3a5	3a6	3a7	3a8	3a9					3b5	3b6
H	3b7			3b9	3b10			3b16				4a2

ISF. 3:
ISF. 4: plate No. 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	4a3	4a4					4a9		4a11			4aB
B		4aD	4aX	4aY	4aZ							4b8
C		4b10		4b12								
D			5a5			5a8				5a12	5a13	5a14
E	5a15					5b2	5b3				5b8	5b9
F		5b12	5b13	5b14		5b16	5b17			5b22	5b23	
G	5b26		5b24				6a4	6aA				6b2
H			6b5									

Read Interval: 00:02:00
Fl : 0.000 - 1000.000

Run Time: 00:29:58
Time: 00:00:00-00:29:58

ISF. 3:
ISF. 4: plate No. 3

	1	2	3	4
A				
B				
C				
D				
E				
F		7aA		
G				
H	7a8			

Read Interval: 00:02:00
Fl : 0.000 - 1000.000

SF. 3:
SF. 4: Further 1 x dilution of a few sample

	1	2	3	4	5	6	7	8	9
A	1b1	3b7							
B	165	3b11							
C	167	5b13							
D	1b20*	5b16							
E	2b5	5b22							
F	2b17								
G	3a3								
H	3b6								

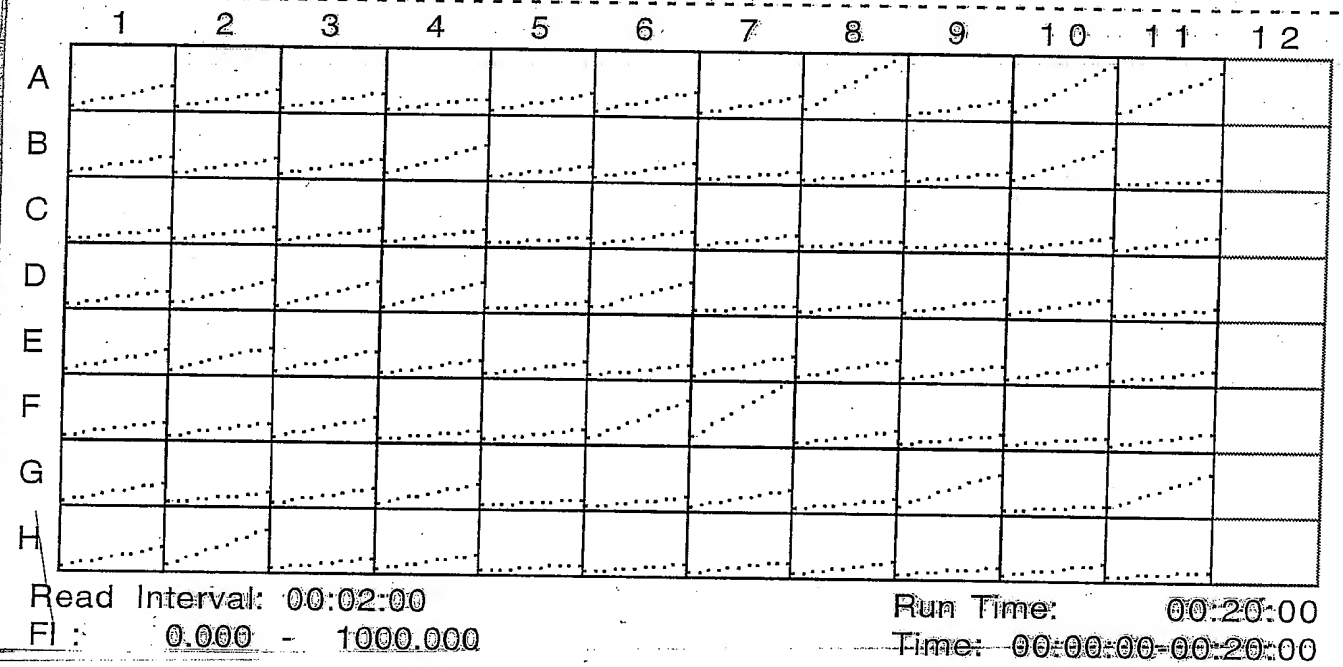
Read Interval: 00:02:00
Fl : 0.000 - 1000.000

Run Ti
Time:

Project (code) Book No: Cont'd from page

Experiment Title [ ] Repeat alls Assay for those with too high reading (with red numbering) of yesterday's experiments.

Dilute 5x of the solution used for yesterday's Assay, use 1.5 ml for each assay.



	1	2	3	4	5	6	7	8	9	10	11	12
A	1a1 16.89	1a3 13.90	1a5 13.04	1a6 9.804	1a7 14.04	1a8 16.44	1a9 13.71	1b5 44.92	1b3 13.02	1b5 39.14	1b7 33.97	
B	1b8 13.21	1b10 11.84	1b12 13.26	1b13 24.49	1b14 9.566	1b15 13.41	1b16 7.184	1b19 8.893	1b20 8.579	1b20* 27.22	1b23 4.855	
C	1b24 7.982	2a1 10.47	2a3 9.789	2a4 10.41	2a14 5.600	2a16 11.05	2a18 8.823	2b1 5.965	2b2 5.442	2b3 9.739	2b5 10.62	
D	2b11 11.24	2b13 19.73	2b16 20.59	2b17 21.02	3a2 6.334	3a3 22.91	3a5 5.853	3a6 9.915	3a7 12.79	3a8 13.22	3a9 6.288	
E	3b5 15.40	3b6 18.74	3b7 16.94	3b9 11.34	3b10 9.949	3b16 9.317	4a2 16.84	4a3 14.69	4a4 10.74	4a9 13.22	4a11 8.365	
F	4a13 11.35	4a15 11.34	4a16 16.02	4a17 7.297	4a22 9.181	4a23 31.19	4b10 47.35	4b13 9.792	5a5 8.264	5a8 7.410	5a12 10.49	
G	5a13 14.42	5a14 8.307	5a15 12.05	5b2 16.12	5b3 5.499	5b8 7.863	5b9 14.70	5b12 8.251	5b13 27.26	5b14 5.649	5b16 28.11	
H	5b17 15.65	5b22 28.87	5b23 8.750	5b26 12.60	5b29 5.594	6a6 7.089	6a8 9.247	6b2 9.937	6b5 6.743	7a8 10.10	7a14 5.212	

These figures are generally lower than expected (or yesterday's reads).

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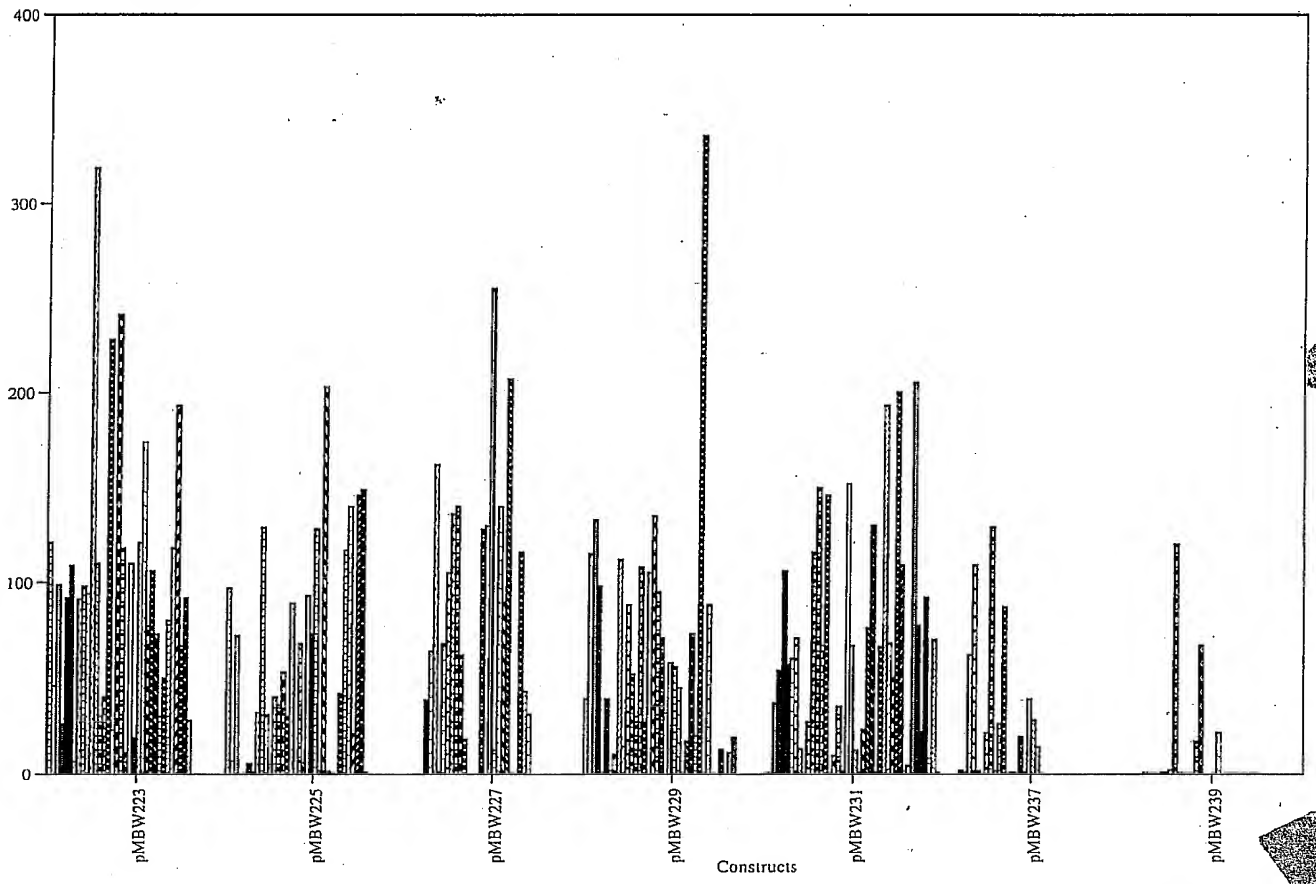
Data #2



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